EXPLORATION OF NOVEL METHODOLOGIES DURING SPAWNING AND LARVICULTURE TO ENHANCE HATCHERY PRODUCTION OF WARMWATER MARINE FISH

A Dissertation

by

ELIZABETH HELEN SILVY

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	Todd D. Sink
Co-Chair of Committee,	Delbert M. Gatlin
Committee Members,	Michael P. Masser
	Robert R. Vega
Head of Department,	David J. Caldwell

August 2019

Major Subject: Wildlife and Fisheries Sciences

ABSTRACT

Aquaculture is vital for supporting the United States (U.S.) seafood production and to meet the growing demand for seafood and lessen reliance on foreign imports. The U.S. is turning to novel technologies and refining methodologies in aquaculture production to meet these demands. Especially, there is a clear lack of warm-water, marine-culture facilities in the U.S.

To address the needs of the growing aquaculture industry, I conducted four separate research trials. The first evaluated the use of a sucrose based time-release excipient in hopes the excipient would have the potential to replace large and invasive hormone implants that are currently used for spawning cobia (*Rachycentron canadum*). The primary objective was to determine if slow-release, cellulose-based spawning peptide implants containing sGnRHa combined with an intramuscular sGnRHa peptide and domperidone injection would synchronize and induce ovulation in captive, sexually-mature female cobia. The second study was a clinical trial to make observations of gross pathology of a novel excipient on red drum (*Sciaenops ocellatus*). The primary objective was to assess the safety and conversely any adverse effects of a sucrose-based excipient when injected into the dorsal musculature or intra-coelomic cavity of red drum. The third was to evaluate methodologies to create gynogenetic female clones of southern flounder (*Paralichthys lethostigma*). The primary objective was to determine which shock treatment (pressure or cold shock) would induce diploid genesis and produce more viable offspring in southern flounder. The final study evaluated the creation of a live-foods-harvesting system for spotted seatrout (*Cynoscion nebulosus*) larvae. The primary

objective was to investigate the feasibility of automated live-food-harvest systems in marine larviculture.

Results showed the use of sGnRHa combined with an intramuscular sGnRHa peptide and domperidone injection to synchronize and induce ovulation in cobia produced eggs within 120 hours after injection. However, 12 hours after egg production, eggs were inspected under a microscope and found to be malformed and displayed signs of fungal infection and no larvae were produced. The induction of ovulation through hormone injection in sexually mature adult cobia was successful.

Results from the clinical trial to make observations of gross pathology of a novel excipient on red drum found survival after treatment was 100% with no signs of primary or secondary bacterial infections. Only 5 of the 20 fish injected showed minor effects of the injections. While this was the first clinical trial using this sucrose-based excipient, it has the potential to replace commonly used hormone implants or injections.

Results to create gynogenetic female clones of southern flounder found a significant ($F = 9.93$, $df = 2$, $P = <0.0001$) difference between the mean egg fertilization/activation rates with the non-irradiated control being different from the irradiated control, irradiated pressure-shock treatment, and irradiated cold-shock treatment. The non-irradiated control demonstrated the highest rate of fertilization and the highest percentage of identifiable late-stage embryos. The irradiated cold-shock treatment and irradiated pressure-shock treatments had similar egg activation rates and embryonic development rates. Pressure-shock represents the most viable method of

creating gynogenetic clones of the female that can then be sex reversed to produce a broodstock consisting of genetically female fish that are phenotypically male.

Results from the evaluation of a live-foods-harvesting system for spotted seatrout larvae indicated the mean number of fish surviving in the control tanks, the enriched-rotifer-monoculture tanks and live-foods-harvesting-system tanks was not significantly ($F = 3.59$, $df = 2$, $P = 0.071$) different. However, the mean size (cm) of control larvae were significantly ($F = 5.99$, $df = 2$, $P = 0.004$) larger than larvae for the enriched-rotifer-monoculture, while larvae in the live-foods-harvesting-system were intermediate in size and not significantly different from any other group. As this was an original system, issues such as mechanical problems were to be expected and further testing with the live-foods-harvesting system may prove its efficiency at replacing hand-cultured live-foods diets for larval marine fish.

In conclusion, all four studies contributed to expanding technologies and methodologies during spawning and larviculture phases of hatchery production. These technologies and methodologies have application to many fish species.

DEDICATION

This dissertation and all of my graduate work is dedicated to my dad, Dr. Nova Silvy and my mom Dr. Valeen Silvy. Thank you for instilling in me a life-long love of learning.

ACKNOWLEDGEMENTS

Thank you to my graduate committee for their endless patience and support throughout my time at Texas A&M University. I owe a great deal to Dr. Todd Sink, my major professor, for everything he has taught me, from building my first system, to how to pour concrete. Thank you for taking a chance on me. Thank you to Dr. Michael Masser who has given me priceless advice and support as well as being a shoulder to lean on. Thank you to Dr. Robert Vega who has always been there for me with a positive word and has facilitated my connection with the Texas Parks and Wildlife Department. Without the aid of the Texas Parks and Wildlife Department, especially the Lake Jackson hatchery at Sea Center, none of this research would have been possible. Specifically, thanks go to Jennifer Butler, Paul Cason, David Abrego, and Jeff Bayer. And thank you to Dr. Delbert Gatlin, whose intelligence and willingness to lend a hand or his even entire lab to aid me in my research has made many of my endeavors possible.

The Gatlin lab at the Aqua cultural Research and Teaching Facility has my whole hearted thanks and appreciation and will always hold a special place in my time at Texas A&M University. Specifically, Xena Ju, Clement de Cruz, Fernando Yamamoto, Alton Burns, Brittney Peachey, and Paul Chen. And thank you, a thousand times, to Brian Ray, a man that I admire and appreciate to no end, for his endless support, patience, and capability.

My appreciation for my own personal lab mates and student workers knows no bounds. Thank you, Elizabeth James, who ran our lab and managed things on a daily basis, as well as becoming one of my closest friends who has loved and supported me

through this entire process. Thank you to John Andrasko, Diego Navaro, Kallie Thornhill, and Isaiah Ringen (for the comedic support).

My thanks to the Department of Wildlife and Fisheries Sciences at Texas A&M University, many of whom have seen me grow from an undergraduate to a graduate student and have always gone out of their way to help and support me. Specially, Kathrine Bradshaw, Lindsay Hutchins, Faegen Lee, Dr. Roel Lopez, Dr. Brian Pierce, Dr. John Tomecek, Dr. Kevin Conway, Royce Robinson, Dawn Miles, Selina Garcia, Dr. Frances Gelwick, Mark Wilder and countless other students, professors and staff who have made my time at Texas A&M University into an unforgettable experience.

Lastly, thank you to my family and friends for their endless love, support, and patience. Thank you to my dad, Dr. Nova Silvy, for the advice, encouragement, love, and countless proofreads. Thank you to my wonderful best friends Emily Dhonau and Dannika Julson for their continuous love and willingness to listen to me rant. And thank you to my boyfriend, Adam Hansley, for your support, intelligence, your strength, and everything you have done and continue to do to encourage me every day.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors Todd D. Sink (advisor), Delbert M. Gatlin, and Michael P. Masser, Department of Wildlife and Fisheries Sciences (Home Department) and Professor Robert R. Vega, Department of Life Sciences, Texas A&M University–Corpus Christi (Outside Department).

Funding Sources

My graduate study was supported by a Graduate Research Assistantship from Texas A&M University AgriLife Extension

This work also was made possible in part by Texas Parks and Wildlife Department under Grant Number 487654 and the National Institute of Food and Agriculture**,** U.S. Department of Agriculture under Grant Number TEXN0077. Its contents are solely the responsibility of the author and do not necessarily represent the official views of Texas Parks and Wildlife Department and the National Institute of Food and Agriculture**,** U.S. Department of Agriculture.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Aquaculture is vital for supporting the United States (U.S.) seafood production, seafood industry related jobs, rebuilding overfished populations, and enhancing coastal resilience. Throughout the world aquaculture provides over 50% of all seafood produced for seafood production, and as the demand for seafood and the need for alternative sources of proteins increases, this percentage will continue to rise (NOAA Fisheries 2018).

The U.S. currently ranks 16th in seafood production with a combined value of $1.45 billion for both fresh and marine aquaculture (NOAA Fisheries 2018). However the U.S. imports 80% of the seafood it consumes with most imported seafood arriving from Asia which holds 89% of the global aquaculture market (NOAA Fisheries 2018). The U.S. is the largest importer of seafood in the world, importing 15% of the worlds seafood worth over $20 billion dollars (FAO 2018). To meet the growing demand for seafood and lessen reliance on foreign imports, the U.S. is turning to novel technologies and refining methodologies in aquaculture production.

One reason for the large number of imports may be due to the lack of onshore marine commercial facilities and research aquaculture facilities. In other countries, where regulations are not as strict, there have been considerable advancements in off-shore, or coastal aquaculture, and those advancements have transferred to on-shore developments in marine aquaculture. Because of the constraints that exist in U.S. coastal waters, the National Research Council suggested the best possibilities for U.S. marine

aquaculture lay inland and offshore (Nelson et al. 1999). Onshore aquaculture has progressed in the U.S. in recent years, especially in the catfish industry, yet many high-value marine species have yet to make the transition to commercial or research aquaculture.

As demand for seafood increases, pressure is placed on wild fish stocks. In 2017, commercial catch in the United States was 4.5 million metric tons of seafood, in Texas alone, commercial catch exceeded 39,788 metric tons, with estimated landings revenue of $250 million (NOAA Fisheries 2018). The pressure put on wild fish populations have had a marked effect on commercial fishing efforts. To support the growing demand, commercial fisherman are targeting less desirable fish populations, fishing further from shore, and harvesting smaller fish. Aquaculture is a sustainable solution to the pressure placed on wild fish stocks through commercial harvest. Stock enhancement programs are designed to boost wild fish populations and cultured seafood directly competes with commercially harvested seafood at market.

Aquaculture not only helps relieve pressure on wild stocks, but serves to generate thousands of jobs in the fisheries field; the Food and Agriculture Organization of the United Nations estimated that 32% of the world's population's livelihoods are supported by aquaculture (FAO 2018). With increases in aquaculture, and advances in culture methods, this number could increase exponentially. Throughout the U.S., there are over half a million jobs supported by the seafood industry, and the development of new practices in aquaculture will create more.

Currently there are 369 finfish species that are farmed throughout the world (FAO 2018). In the past 10 years the number of cultured species has increased by 26.7% (FAO 2018). This established a demand for seafood that has directly affected the direction of aquaculture towards investigations and development of novel species for various culture facilities. The U.S. primarily produces five principal species (catfish [(*Ictalurus* spp.], trout [*Salmo* spp.], salmon, tilapia [*Oreochromis* spp.], and hybrid striped bass [*Morone chrysops* x *M. saxatilis*]; APHIS 1995).

In 2013, the U.S. Department of Agriculture's Aquaculture Census (USDA 2013) reported there were 3,093 aquaculture farms in the U.S. Divided by category, there were 1,296 food fish farms, 282 sport fish farms, 166 baitfish farms and the rest were crustacean, mollusk, ornamentals and miscellaneous aquaculture farms (USDA 2013). Of these farms, catfish, trout, and tilapia were the most numerous with 695, 359, and 181 farms, respectively (USDA 2013). Warmwater marine species such as red drum (*Sciaenops ocellatus*) and flounder (*Paralichthys sp.)* have considerably lower numbers of farms 3, and 7, respectively. When these data were reviewed, there was a clear lack of warmwater-marine-culture facilities in the U.S.

Aquaculture is still a relatively new science, with its rise in prominence occurring in the 1970's (White et al. 2004) and as the data indicates, only a small percentage of fish have been introduced to the market through aquaculture. To fill the widening gap between supply and demand, new methodologies must be investigated and there must be refinement of technology, with research pertaining to larviculture, maturation, spawning

of novel species undertaken, and innovative solutions to current bottlenecks in culture remedied.

To address the needs of the growing aquaculture industry, I conducted four separate research trials. Chapter II evaluates a combination of hormone implants and injections used to induce ovulation in sexually mature female cobia (*Rachycentron canadum*) which is a relatively novel species to aquaculture and methodologies in spawning have yet to be refined. The original purpose of this study was to produce fertilized cobia eggs to grow out using a novel live-foods-harvesting system. Due to complications with the collection of both genders of sexually mature broodstock to produce fertilized eggs and lesions that led to mortality in broodstock, the goals of this project were altered and subsequent research was undertaken to address these issues. Mortality caused by hormone implantation in the second study led to the testing of a sucrose based time-release excipient (Chapter III) in hopes the excipient will have the potential to replace large and invasive hormone implants that are currently used for spawning. Chapter III tests the effects of a novel excipient on red drum (*Sciaenops ocellatus*). Chapter IV addresses the bottleneck of a skewed gender bias in southern flounder (*P. lethostigma*) culture by evaluating methodologies to create gynogenetic female clones. Chapter V addresses the original goals of the cobia study, through the replacement of the economically costly and time consuming process of live-foods cultivation to larval marine species, through the creation of a live-foods-harvesting system. Fertilized spotted seatrout eggs (*Cynoscion nebulosus*) were used in place of

cobia eggs in this study. Chapter VI summarizes the conclusions of the four research projects.

CHAPTER II

THE USE OF MATURATION PEPTIDES TO INDUCE AND SYNCHRONIZE OVULATION IN CAPTIVE, SEXUALLY-MATURE, FEMALE COBIA

Introduction

Cobia (*Rachycentron canadum*), also referred to as ling and lemonfish, is a popular recreational sportfish, an internationally, commercially cultured food fish, and has tremendous potential as a domestically cultured species in the United States. Cobia is a high priority species for aquaculture in the Gulf of Mexico, the Southeastern US coast, the Caribbean, and the subtropical/tropical waters of the Atlantic coast of South America (Benetti et al. 2007). Due to low supply, high market demand in price ($37.40/kg of fillet), fast growth rates, relatively low larval mortality compared to other marine species, and good feed conversion rates, cobia has been a fast growing sector for aquaculture in Asian and South and Central American countries such as Taiwan, South Korea, China, India, Panama, Brazil, Belize and the United States (Su et al. 2000, Liao et al. 2004, Benetti et al. 2008).

Cobia is a prime candidate for aquaculture for commercial food-fish production as well as stock enhancement programs to increase wild stocks for harvest. Little data exists on the population status of cobia, because they are primarily a recreational fish due to their solitary, pelagic life history that makes commercial harvest economically unfeasible, resulting in a limited commercial industry. In the limited commercial industry, it is easy to track the decline of cobia populations. In 2014, 18,143.7 kg of

fresh and frozen fillets were harvested for the commercial market, down 3,638.7 kg from 2013 (NOAA Fisheries 2015). Recreational landings, either purposeful, or as by-catch, comprised 90% of total landings. In 2013, 54,431.0 metric tons of fish were recreationally harvested (NOAA Fisheries 2013). The System for Electronic Document Analysis and Retrieval (SEDAR 2013) estimated total abundance and recruitment of cobia was below average following an increase in stock abundance in the early 2010s. Estimated stock abundance declines were due in part to recreational and commercial by-catch (SEDAR 2013). Stock enhancement programs for cobia in Mississippi, South Carolina, and Florida have shown varying degrees of success to bolster total abundance and recruitment (SCDNR 2015). Between 2001 and 2009, South Carolina's Department of Natural Resources released 64,768 cobia fingerlings (SCDNR 2015). These numbers are significantly lower than that of red drum (*Sciaenops ocellatus*) stock enhancement programs. More than 33 million red drum were released in 2010 (SCDNR 2015) and over 735 million have been released in Texas as of August 2018 (Robert Vega, Texas A&M University–Corpus Christi, Texas; personal communication, 2019).

The earliest known attempt at spawning cobia in captivity occurred during the 1970s in North Carolina, in which researchers harvested gametes from wild caught broodstock and failed to produce juveniles from the larval stage (Hassler and Rainville 1975). Further research efforts have been made to spawn cobia in captivity in Florida, Mississippi, South Carolina, Texas, and Virginia (Kaiser and Holt 2005). Many of these efforts attempted to induce spawning using injectable human chorionic gonadotrophin (hCG) or slow-release implants (salmon gonadotropin releasing hormone analogues,

sGnRHa; Franks et al. 2001). Several attempts to spawn cobia were successful using hCG or luteinizing hormones, but failed to optimize larval production and resulted in poor survival (Hassler and Rainville 1975; Kaiser and Holt 2005).

In the early 1990s, researchers at the University of Texas began rearing wild caught juvenile cobia in captivity to maturity. Researchers documented the first volitional spawning of cobia in 2002 using water temperature and photo-period manipulation (Arnold et al. 2002). However, only a single pair of a larger group of cobia was documented to spawn. Volitional spawning brought on by only environmental conditioning has proven to be sporadic and unreliable leading to eventual abandonment of some U.S.-based cobia research programs. Most successful spawning of cobia has been due in part to induced spawning of broodstock through spawning peptide application, or by capture of mature spawning adults during the natural spawning season (Dodd 2001, Franks et al. 2001, Arnold et al. 2002). Successful spawning of adult cobia have been accomplished using the implantation of pelleted hCG (275 IU/kg BW; Franks et al. 2001), and with hormonal injection of 10 µg/kg luteinizing-releasing hormone analog (LHRHa, des-Gly^{10}, and D-Ala^{6}; Sigma-Aldrich, Singapore; Nguyen et al. 2010), but only in small numbers.

As university researchers and commercial hatcheries investigate the potential of novel species for culture, there is a need for research to investigate the ability of salmon gonadotropin-releasing hormone (sGnRha) implants and injections combined with environmental conditioning to induce spawning in captive cobia broodstock. Salmon gonadotropin-releasing hormone implants are commonly used to induce oogenesis and

spermatogenesis in various marine and freshwater species such as Atlantic salmon (*Salmo salar*), Chinook salmon (*Oncorhynchus tshawytscha*), common snook (*Centropomus undecimalis*), Atlantic croaker (*Micropterus undulates*), Arapaima (*Sudis gigas*), common carp (*Cyrprinius carpio carpio*), and Atlantic cod (*Gadus morhua*) with high degrees of successful spawning (Garber et al. 2009, Sink et al. 2010, Ibarra-Castro et al. 2011, Vazirzadeh et al. 2011, Torati et al. 2017). The use of sGnRha peptide implants could alleviate bottlenecks preventing cobia production on a commercial scale by increasing cobia spawning success, eliminating seasonal availability, and establishing a steady supply. Improved spawning success also could alleviate capture pressure on wild stock through stock enhancement programs.

During the course of my investigations, a Ralgun® implanter was used to administer Ovaplant® (Syndel International Inc., Qualicum Beach, British Columbia, Canada) implants, just as it has been used in numerous other studies (e.g., Tvedt et al. 2001, Ingram et al. 2005, Garber et al. 2009, Broach et al. 2015, Kuradomi et al. 2017). However, due to the large size of cobia broodstock, limited dosage range of OvaPlant® implants, and the total number of implants required to achieve the minimum effective dose, the Ralgun® implanter used to deliver the OvaPlant® implants was noted to lead to secondary infections and deaths due to numerous large holes created in the fish during implant delivery. Due to the incidence of infection and disease, further investigation into alternative, slow-release delivery systems for sGnRHa was conducted to prevent infections of the broodfish. Potential solutions to these issues became the focus of other chapters in this dissertation. To achieve the potential benefits of sGnRHa spawning aides

for cobia spawning, I conducted a study to determine the feasibility of cobia larval production using sGnRHa spawning aids such as Ovaplant® and Ovaprim®.

Objectives and Hypotheses

The primary objective of my study was to determine if slow-release, cellulose-based spawning peptide implants containing sGnRHa (Ovaplant®) combined with an *intramuscular (*IM) sGnRHa peptide and domperidone (dopamine inhibitor) injection (Ovaprim® Syndel International Inc., Qualicum Beach, British Columbia, Canada) could synchronize and induce ovulation in captive, sexually-mature female cobia. The null research hypothesis was that sGnRha implants have no effect on synchronization and inducement of ovulation in female cobia.

Methods and Procedures

Broodstock Collection

Sexually mature adult cobia (n = 5, mean length 0.873 m, mean weight 7.18 kg) were captured from the Western Gulf of Mexico cobia population using hook and line. Broodstock acquisition trips were conducted on a 7.62 m TransCat boat, captained by a local guide. Three collection trips were made, with cobia capture between 11.3 and 27.4 km offshore near oil platforms (Fig. 2.1). The boat first circled the rig and then lines were dropped and jigs were used to entice cobia. Cobia were captured with traditional hook and line using jigs and live croaker purchased at a local bait shop. Other baits such as frozen ribbon fish and crab were used, but did not result in cobia capture. Jigs were the initial bait that attracted cobia and when the first fish was hooked and brought to the

surface, the schooling nature of the species brought companion fish to the surface. A live croaker on an unweighted line was then thrown in front of companion fish, resulting in multiple fish being captured at a single oil rig. During this process and due to the unreliable nature of hook and line fishing and user ineptitude, some fish broke off the line or escaped capture. Fish were then guided into a large net and brought aboard the boat and the hook was removed. Permits for collection of cobia broodstock from state waters were obtained from the Texas Parks and Wildlife Department (SPR-0718-210). Hook and line was the most efficient and least stressful manner to capture large solitary cobia in the wild and resulted in the least amount of physical damage to the fish compared to other capture methods such as nets, trawls, and long-lines. Cobia broodstock were immediately transferred into a double walled, insulated, 833-L shipping tote that contained 662-L of ambient seawater (approximately 32–24 g/L salinity) located on the flat front deck of the boat. Supplemental oxygen was provided to the shipping tote at 2-L/minute (Regulator; Roscoe Medical, RMI-15H CGA-540 H Regulator, 0-9 LPM Compass Health Brands, 6753 Engle Road Middleburg Heights, Ohio) from a 6.23-cubic meter compressed oxygen cylinder and fine pore, porcelain air stones (69.9 cm x 8.5 cm, Point Four™

Figure 2.1. Area in the Gulf of Mexico where cobia were obtain for this study.

Micro Bubble Diffusers, Pentair Aquatic Eco-Systems, Inc., Apopka, Florida). Multiple fish were collected before the boat returned to shore.

Broodstock Transport

Cobia broodstock were transported to the Aquacultural Research and Teaching Facility (ARTF; 13950 FM 60 East, Somerville, Texas) in a 1,325-L-insulated-hauling tank. The insulated hauling tank was filled with 33 ppt seawater prior to onboard collection and temperature was uncontrolled, but recorded at 25°C. Hauling tanks were provided oxygen from a 6.23-cubic meter compressed oxygen cylinder and dissolved oxygen (DO) was provided at 2-L/minute. These values ensured acclimatization between

boat tank and hauling tank. Vitalife® (Syndel International Inc., Qualicum Beach, British Columbia, Canada) slime coat was added to the tank water to prevent mucus build up and surface slime that could potentially limit oxygen consumption. These procedures were intended to limit stress on fish.

Broodstock Acclimatization

When broodstock arrived at the ARTF, temperature, salinity, and DO were assessed in the hauling tank and the recirculating system. On one occasion there was a discrepancy between temperature of the hauling tank (25°C) and the recirculating system (28°C) and water transfer was made to acclimatize the fish between systems. Approximately 189.3 L were transferred from the hauling tank using a 2.45-cm pump to the recirculating system and then another 189.3 L was transferred using a 2.5-cm pump from the recirculating system to the hauling tank to moderate the temperature difference. After this procedure, I waited 15 minutes, tested temperature, and then repeated four times before temperatures were within one degree in each system.

Broodstock were then transferred to a black colored 1,000-L tank, with oxygen provided from a 6.23-cubic meter compressed oxygen cylinder at a rate of 2-L/minute, for a formalin dip to rid fish of parasites. Fish were transferred one at a time and weight and length were recorded. After 2 minutes in the formalin dip (25 mg/L), broodstock were moved to a 4,429-L recirculating tank system that was 27°C, 33 ppt and had a DO of 6.4 mg/L. Compressed oxygen at a rate of 2-L/minute was provided for 48 hours after transfer into the recirculating system using a compressed oxygen cylinder and fine pore, porcelain air stone (69.9 cm x 8.5 cm, Point Four™ Micro Bubble Diffusers). Fish

were acclimatized to a photoperiod of 14 h of light and 10 h of dark with water temperature ranging from 26–30°C to simulate natural spawning conditions. System salinity was maintained at 28–32 ppt with artificial sea salt (Red Sea Salt, Red Sea North America, Houston, Texas). Dye (Aquashade®, Arch Chemicals, 501 Merritt 7 Norwalk, Connecticut) was added to the water of the tank to more easily acclimatize the fish to a lighter tank environment.

Broodstock Care and Feeding

Broodstock were kept in a 4,429-L recirculating tank, at a photoperiod of 14 h of light and 10 h of dark photoperiod and at a water temperature of 26–30°C to simulate natural spawning conditions for the duration of the trial. The recirculating system consisted of one 4,429-L tank. The system includes a sump tank filled with two sacks of bioballs/filtration media for water collection, a 2-horsepower pump (Hayward® MaxFlo XL 2 HP Dual Speed Pool Pump, One Hayward Industrial Drive Clemmons, North Carolina), a Arias 4000© (Pentair Aquatic Eco-Systems Inc. 2395 Apopka Blvd. Apopka, Florida) bead filter, a 25-µm Water Co.© filter (Water Co.®, Augusta, Georgia), and a Jebao© PU-36 UV clarifier (Jebao®, Dongsheng, Zhongsha, Guangdong, China). One half of the water that exited the UV filter was returned to the sump tank. The other half of the water that exited the UV filter returned to the tank at an angle encouraging uni-directional water flow in a circular pattern. The tanks were set up with a venturi drain style standpipe, covered in large mesh that let waste water flow into a drainage pipe that deposited waste water into the sump tank for filtration (Fig. 2.2) Aeration was

applied using four air stones from an external blower (Whitewater® Regenerative Blower Pentair Aquatic Eco-Systems Inc. 2395 Apopka Blvd. Apopka, Florida).

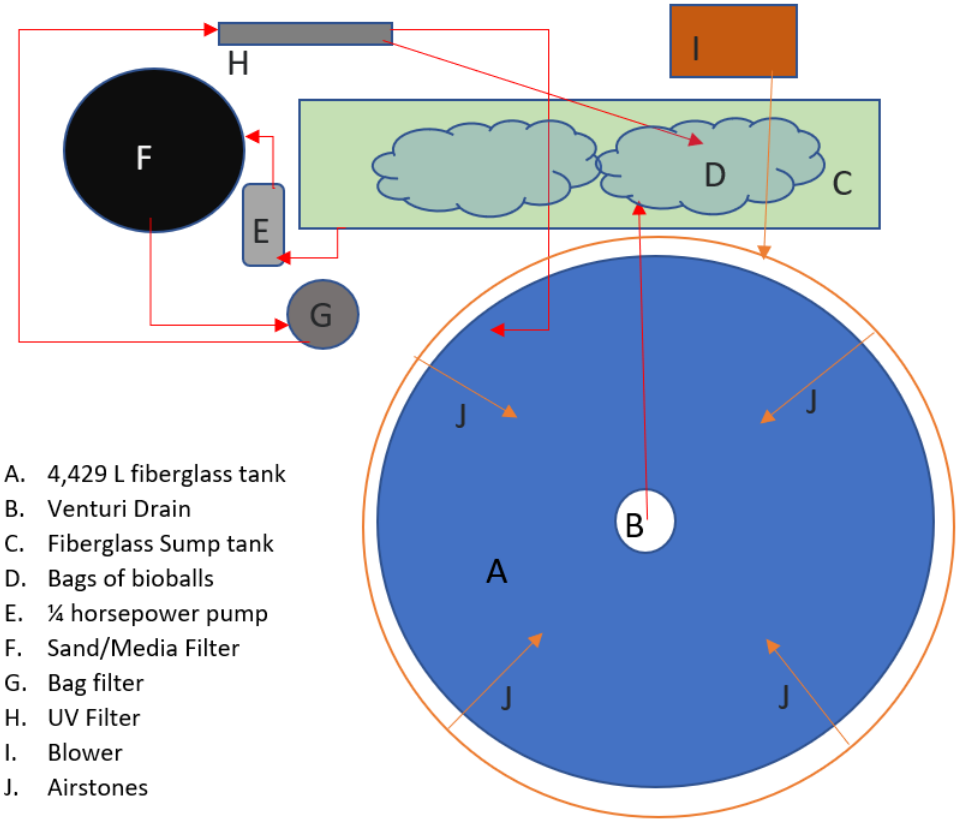


Figure 2.2. A diagram of the recirculating broodstock system used for this research.

Broodfish were fed a mixture of frozen shrimp trawl bycatch, freshwater lake survey bycatch (Texas Parks and Wildlife Department, District 3 Fisheries Biologist Office, Sommerville, Texas), and live baitfish (Bait Barn Fisheries, Bryan, Texas) daily until satiation. Temperature, dissolved oxygen, salinity, and pH were monitored and recorded

daily. Temperature was maintained at 26–30°C using heaters (Finnex®, HMA-S 100 Watt Titanium Heater, 360 Aquatics, 5710 Brittmoore Rd #9, Houston, Texas) located in the sump tank of the system. System salinity was maintained at 28–32 ppt with artificial sea salt (Red Sea Salt, Red Sea North America, Houston, Texas). Total ammonia nitrogen, un-ionized ammonia, and nitrite were determined and recorded bi-weekly. After 1 week in the recirculating tank system it was observed that fish were prone to jump out of the recirculating tank system, thus a large black mesh shade was then placed over and covered 90% of the tank system.

Hormone Implantation

After a 3-week acclimatization period to the system, fish were removed from the tank, sedated in an isoeguenol bath (Thermo Fisher Scientific Chemicals, Inc., Ward Hill, Massachusetts), and weight and length of each fish was recorded to calculate appropriate spawning peptide dosages. Each broodfish was administered 250 µg Ovaplant® hormone implants in the dorsal musculature anterior to the dorsal fin to achieve a final dosage of >50 µg/kg of body weight, and injected with Ovaprim® in the dorsal musculature on the opposite side of the implants anterior to the dorsal fin at a dosage of 25 µg/kg of body weight. These are standard dosages for spawning large fish determined from the product labels and were discussed with and confirmed by the product manufacturer (Dr. Peter McKenzie, Senior VP Product Management, and Dr. Katie Haman, DVM, Director for Spawn Products, Syndel, Ferndale, Washington). After injection, the fish were immediately returned to the tank and allowed to spawn volitionally within the tank.

Egg Collection and Ovulation Assessment

An egg harvester made of 420-µm mesh was placed under the tank outflow pipe and monitored for eggs from 3 to 7 days, post-injection. Egg collection chambers were checked every 6 hours for presence of eggs. Date and time of egg collection was recorded when eggs were obtained from the collectors. When eggs were present, they were collected from egg collector using a 1,000-mL pitcher. The total quantity of eggs was extrapolated from 1 mL egg counts taken from 1L collections out of the egg collection chamber. The number of 1L collections also was recorded. Visual observations were made of egg size, quality, and clarity.

One thousand eggs per liter were incubated in each of 16, 113-L recirculating incubation tanks. The incubation system included a sump tank for water collection, a 2-horsepower pump (Hayward®), a sand filter (Arias 4000©), a cannister filter (Water Co.©), and a UV filter (a Jebao©). One half of the water that went through the system exited the UV filter and was returned to the sump tank. The other half of the water that exited the UV filter returned to the tanks. The tanks were set up with an internal standpipe covered in 250-micron mesh that led to an external standpipe that maintained water level and let waste water flow into drainage pipes that deposited waste water into the sump tank for filtration. Temperature was controlled by a chiller/heater (Aqualogic®, 9558 Camino Ruiz, San Diego, California). Temperature was maintained at 26°C, salinity maintained between 28–32 ppt, and DO was kept above 4 mgL. Incubation tanks were set for a 12-hour light and 12-hour dark cycle schedule. Aeration was

maintained at a low level for the duration of the experiment. Animal Use Protocol 2016-0279 was followed for this experiment.

Data Analysis

Statistical analysis of this study was not possible due to the limitations of equipment, facilities, and numbers of broodfish necessary to have replicates for statistical analysis. This was extremely common for this type of study in which only small populations of large broodfish can be obtained and safely cultured by most research and commercial facilities. These types of studies are typically of more importance to biological and commercial significance than statistical significance. This was exemplified by other studies of this nature including published studies by the University of Texas in which a single spawning event by a single pair of cobia broodstock was publishable due to its significance to the culture and potential production of this species (Kaiser and Holt 2005, Franks et al. 1999). Therefore, this study represents an observational study in which biological and commercial significance, in the form of successful induction of ovulation and ability to obtain cobia eggs, was the criteria used to evaluate the success of the study. Obtaining eggs from a successful ovulation event was considered to be a positive test, while failure to induce ovulation or to obtain eggs was considered failure of the treatment.

Results

A total of five cobia was used in this study. This was comparable to other studies that have used the same number of fish or less (Franks et al. 2001, Weirich et al. 2006).

Cobia captured for this study averaged 7.18 kg in weight and 0.873 m in length and hormone quantity was correlated to weight (Table 2.1). A total of 14 Ovaplant® hormone

Table 2.1. Length, weight, and hormone injection information for individual cobia used in this research.

Fish	Length (m)	Weight (kg)	Dosage of Ovaplant® Administered (# of implants)	Dosage of Ovaprim® administered (mL)
1	0.635	4.6	2	1.15
2	1.09	9.0	3	2.25
3	0.865	7.4	3	1.92
4	0.914	7.5	3	1.87
5	0.862	7.4	3	1.92
Average	0.873	7.18		
Total			14	9.11

implants and 9.11 mL of Ovaprim® was used on the five fish. Immediately following hormone implantation, fish were lethargic and had to be manually held in oxygen flow for 1–5 minutes before resuming normal behavior. Two days after implants were administered lesions were visible at injection sites in two of the cobia.

Approximately 120 hours after injection at 0800 hours on 30 July 2018, eggs were observed in the egg collector. It was assumed that eggs were spawned overnight on the 29 July. Eggs were collected for 2 hours from egg collection chamber and eggs were i

observed floating in the broodstock tank. The cobia were observed to be circling the tank during egg collection. Water temperature was 28°C and salinity was 29 ppt at the time of egg collection. Eggs were small, approximately 1.5 mm in diameter, milky white in color and did not display any observable clarity. A large percentage (~80%) of the eggs was resting on the bottom of the collection chamber while the rest were floating on or near the surface of the water column (Fig. 2.3).

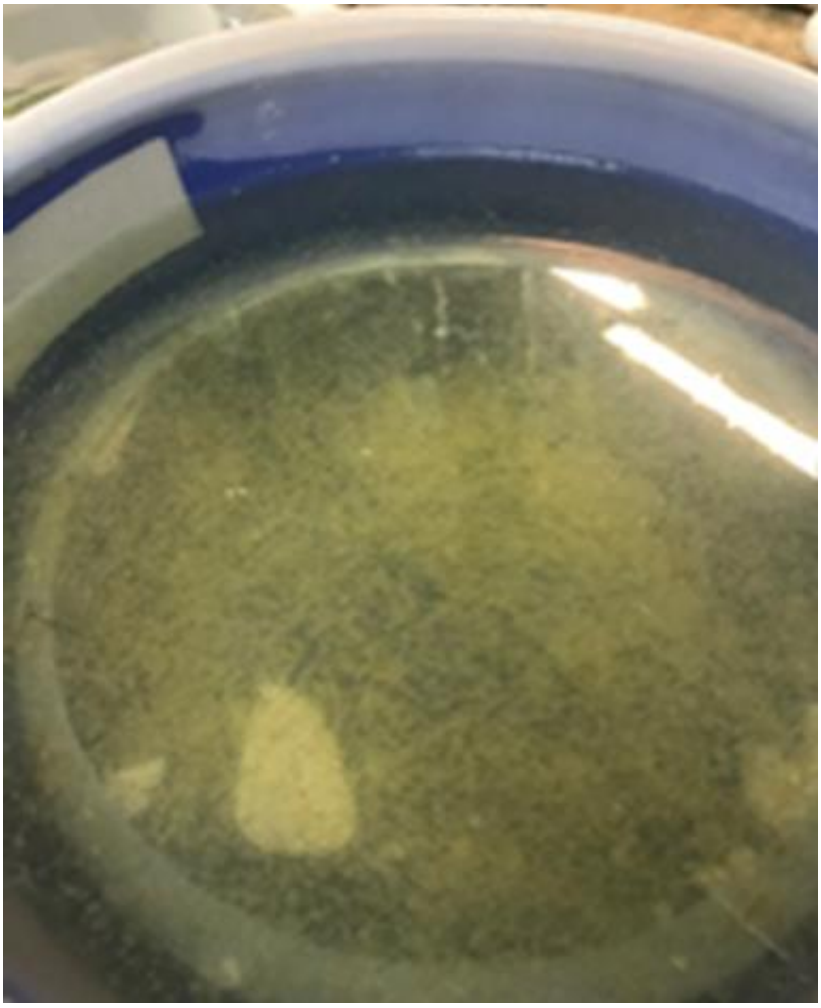


Photo of eggs collected from cobia 120 hours post-injection.

There were approximately 330 cobia eggs per milliliter and 12,000 ml of the eggs were collected. Extrapolating on this enumeration indicated a total of 4 million eggs were produced (eggs from females combined). I was unable to determine the number of eggs spawned per female. Eggs were divided equally and placed into 18 incubation tanks with light aeration and 100% daily water exchange. Mean batch fecundity was enumerated and estimated to be 800,000 eggs per female based on the assumption that all of the fish were female and ovulated.

Twelve hours after estimated egg production, eggs were inspected under a microscope. Eggs were malformed and displayed signs of fungal infection. The specific fungal infection was not identified. All five fish survived the spawning event, but two were clearly sick or infected and displayed large dermal lesions located at injection sites (Fig. 2.4). These fish were lethargic and rested on the bottom of the tank. These two fish were euthanized after the spawning event.

Figure 2.4. Dermal lesions observed following a post-spawning event.

Discussion

The results of my study demonstrate the combination of sGnRHA Ovaplant® implants and Ovaprim® injections were successful in inducing ovulation in female cobia under natural spawning season photoperiod and temperature without intensive photo-thermal manipulation. This was similar to previous studies that used photoperiod and/ or hormonal manipulation to induce ovulation (Franks et al. 2001, Arnold et al. 2002, Kilduff et al. 2002). Biesiot et al. (1994) and Franks et al. (2001) used human chorionic gonadotropin (HCG) injected at 275 IU/kg of body weight in female cobia to produce fertilized eggs after about 42 hours post-injection. Nguyen et al. (2010) used injection of 20 µg/kg LHRHa and egg production was observed within 1-hour post-injection. This differs from my observations in that egg production in my study was observed 120 hours post-injection. This could potentially be due in part to lack of males, whereas males were identified and present in both of the aforementioned studies. The lag between injection and spawning also could be attributed to the time release nature of Ovaplant®

In prior research by Arnold et al. (2002), Kilduff et al. (2002), and Benetti (2003), cobia were spawned successfully using rigorous photothermal regimes. Water temperature manipulation as the only form of induce spawning manipulation has been proven to induce spawning in cobia (Steiglitz et al. 2012) and other species such as red drum (*Sciaenops ocellatus*) (Arnold 1988) and Nassau grouper (*Epinephelus striatus*; Tucker et al. 1996). This was a viable manipulation to induce spawning, but was not practical at a large commercial scale; whereas, hormonally induced spawning using

sGnRHa, hCG, or LHRHa allows for out of season spawning without intensive temperature control.

Batch fecundity in my study was comparable to other small-scale hormone-induced-spawning studies. Franks et al. (2001) observed 3.2 million eggs from two females 42 hours post-injection. While my results are slightly lower, if calculated by eggs per female, this could be attributed to the fact that Franks et al. (2001) included a sexually mature male in their study that could have induced ovulation or there was a smaller lag time between injection and the ovulation event. Previous research conducted by spawning cobia without hormones indicate higher batch fecundity. Weirich et al. (2006) used no hormone injection and stocked three male fish with two female fish and had a mean batch fecundity of two million eggs per female. This was higher than my observed batch fecundity and could be due in part to intense photothermal manipulation used to induce spawning.

In my research, eggs were not analyzed for a specific type of fungal infection. Fungal infections in pelagic finfish species eggs can be attributed to a multitude of fungi such as *Achyla polyandra*, *Saprolegnia ferax, S. parasitica , S. diclina, S. australis*, *S. furcata*, *S. hypogyna*, and *S. unispora* (Fregeneda-Grandes et al. 2007, Czeczuga et al. 2005, Vega-Ramirez et al. 2013). The fungal infection in my research was thought to be caused by a lag in egg production and egg evaluation, improper water filtration, damage during egg collection in the collection chamber, or fungus present in the sump tank where the egg collection chamber was located. The common remedies to fungal infections include a disinfectant dip in 5% iodine immediately after egg collection,

and/or hydrogen peroxide treatment (Arndt et al. 2001, Barnes et al. 1998). Preventative care, adequate nutrition, prophylaxis and probiotics can all contribute to the success of healthy egg production.

Also noted were the presence of lesions caused by the repeated injections with the Ralgun® implanter that was necessary to achieve the desired dosage of Ovaplant®. Lesions were observed in two of the five fish injected at the injection site. A secondary bacterial infection led to the deterioration of the dermis and sub dermal musculature and eventually mortality. Negative side effects of Ovaplant® implantation have been observed in other species. Sink et al. (2010) found that post-implantation of Ovaplant® in Atlantic croaker (*Micropogonias undulates*), injected female fish became lethargic and did not resume normal swimming behavior and mortality occurred in three of the 15 implanted fish. Hill et al. (2009) found that negative effects of Ovaprim® injection included redness and bruising at the injection site and stemmed from degradation of water quality while holding and handling fish. Multiple studies have used Ovaplant® and Ovaprim® to induce gonadal maturation and spawning and have reported no adverse effects (Tvedt et al. 2001, Ingram et al. 2005, Garber et al. 2009, Broach et al. 2015, Kuradomi et al. 2017). It was presumed the lesions and subsequent bacterial infections that led to lethargy were caused by repeated hormone injections needed to achieve the appropriate dose per body weight of Ovaplant®. Secondary bacterial infections could be associated with the nature of bacteria build up in a recirculating system or bacteria found naturally on the dermis of the fish. Further investigations need to investigate new methodology for the delivery of sGnRHa. A less invasive injection procedure would

tamper many of the adverse effect caused by the use of a Ralgun® implanter as well as the need for multiple injections to meet the required dosage.

While my research was considered biologically and commercially successful from the predetermined standards evaluation, subsequent repetitions of the trial and further studies using sGnRHa implants and injections should investigate stage of sexual maturation and gender prior to injection. The inclusion of a known male in spawning trials may be pertinent or just a coincidental effect on successful spawning behavior. However, known males were identified and included in recorded successful spawning trials (Franks et al. 2001, Weirich et al. 2006, Benetti et al. 2007). To assess stage sexual maturity and gender, ovarian samples should be aspirated from the fish prior to injection (Franks et al. 2001). Weirich et al. (2006) sedated fish prior to introduction in tanks and ponds and applied pressure to the abdomen of the fish to detect presence of running milt to identify males, while females were subjected to an ovarian biopsy to determine oocyte stage of development and size. These methods do have adverse effects such as exposure to harmful bacteria and increased handling time which was shown to increase stress and elevate cortisol levels which can delay or prevent successful spawning (Sink et al. 2010).

Alternatively, given the cobia in the Western Gulf of Mexico spawn every 7–12 days (Brown-Peterson et al. 2001), it should be noted the specimens used in my research should all have been sexually mature, due to size, and in at least the early stages of previtellogenesis, due to collection time correlating with spawning season, and the injection of sGnRHa aided in successful spawning. While timing aided in the successful spawning event, to elucidate out of season spawns, sexual maturity and gender must be

assessed before injection. There should be further investigation into out-of-season spawning based exclusively on hormone injection.

As with any cultured species, the success of spawning was dependent on the health and sexual maturity of the species to be spawned. In further studies a combination of photothermal manipulation following guidelines successfully used by Benetti (1997) and Benetti et al. (2001) as well has spawning peptide injection should be used to ensure sexual maturity and prime fish for successful spawn. The combining of both hormone injection and intensive photothermal manipulation can prime fish for spawning in multiple effective ways. The use of sGnRHa has been proven to effectively induce ovulation in early stage fish and has been effective at producing successful spawns in multiple species of fish (Tvedt et al. 2001, Ingram et al. 2005, Garber et al. 2009, Broach et al. 2015, Kuradomi et al. 2017). Environmental manipulation integrates natural physiological functions, such as spawning, with natural environmental cycles thus priming fish for favorable spawning conditions. Water temperature has been demonstrated as the key environmental manipulation to induce off-season cobia spawning (Steiglitz et al. 2012). The key to successful spawning of cobia was a combination of hormonal injections that progressed fish that may have been in early stages of ovulation, as well as the photothermal manipulation that provided the proper environmental cues for successful spawning.

CHAPTER III

EVALUATION OF SAFETY AND GROSS PATHOLOGY OF SUCROSE BASED EXCIPIENT INTENDED TO DELIVER TIME-RELEASED SPAWNING PEPTIDES IN MARINE BROODFISHINTRODUCTION

Introduction

Controlled reproduction in captivity is essential for the continued expansion of aquaculture. As natural fish populations decline, there is an increased effort into research and development of novel technologies for induced spawning (Rottmann et al. 1991, Zohar and Mylonas 2001, Murugananthkumar et al. 2017). In many fish species induced spawning can be achieved by manipulating water temperature, photoperiod, spawning cycle or other environmental conditions (Colura et al. 1991, Smith et al. 1999, Mylonas et al. 2010, Stieglitz et al. 2012). However, many fish species that have great economic significance in aquaculture do not reproduce spontaneously in captivity, or naturally reproduce under conditions that are not easily recreated in a hatchery setting (Lee et al. 1986*a*, Rottmann et al. 1991, Lee and Ostrowski 2001). Hormonal manipulations are the only reliable way to produce fertilized eggs and often used to promote efficiency in egg or milt production (Rottmann et al. 1991, Lee et al. 1988). In addition to breeding desirable fish species, induced spawning can be used to produce hybrids and sterile polyploid fish, produce monosex populations, synchronize reproduction of large numbers of fish for simultaneous spawning, produce larvae outside of the normal

spawning season and maximize survival of larvae in controlled hatchery conditions (Rottmann et al. 1991, Donaldson 1996, Migaud et al. 2013).

Hormonal manipulations in fish reproduction have been in use for the past 80 years (Tamaru et al. 1989, Rottmann et al. 1991, Lee 1997). One hormonal manipulation is the injection of crude extracts of the pituitary gland (PE) of mature fish (Houssay 1931, von Ihering 1937). Another is the injection of exogenous luteninizing hormone (LH) that directly affects the gonads (Mylonas et al. 2010). In more recent years, synthetic agonists of gonotropin-releasing hormones (GnRHa) that act at the level of the pituitary to release LH have predominantly been used to induce spawning (Mylonas et al. 2010, Hoga et al. 2018), as well as human chorionic gonadotropin (HCG; Lam 1982, Zohar and Mylonas 2001). Purified gonadotropins that stimulate ovaries and testes also are used (Schally 1978). Most of the aforementioned hormone injections can be used along with dopamine blockers which enhance potency of LHRHa (Rottmann et al. 1991, Venturieri and Bernardino 1999, Almeida 2013).

Currently, the only commercially available slow-release spawning peptides to induce gonad maturation and ovulation in warm water marine finfish are configured as cellulose-based implants (e.g., Crim 1985, Lee et al. 1986*b*, Sherwood et al. 1988, Tvedt et al. 2001, Ingram et al. 2005, Garber et al. 2009, Broach et al. 2015, Kuradomi et al. 2017). These implants are several millimeters in diameter (up to 3.5 mm), and depending upon dosage and composition, have lengths up to five times their diameter. They are implanted using Ralgro® (Merck & Co. Inc. 2 Giralda Farms, Madison, New Jersey) pellet injectors or Ralgun® implanters (Syndel International Inc., Qualicum

Beach, British Columbia, Canada) originally developed for injecting large Ralgro® implants into cattle and other livestock. Implanters create large diameter holes (needle outside diameter of 4.7625 mm) in the dermis compared to needles used with aqueous hormones (23-gauge needle outside diameter of 0.6414 mm), thus exposing fish to infection by the numerous pathogenic bacteria and fungi present in aquatic environments and eventually causing mortality (Hansen and Olafsen 1999, Olafsen 2001, Hill et al. 2009).

Despite the large size of implants, relatively small dosages are available in commercial implants. For example, the maximum dosage available for Ovaplant® implants (Syndel International Inc., Qualicum Beach, BC, Canada) is 250 µg of salmon gonadotropin releasing hormone analogue (sGnRHa), and the effective dosage in fish is 50 µg sGnRHa/kg body weight. This means that large broodfish frequently require several implants be administered per fish, creating multiple large holes in the dermis to deliver the appropriate dose of peptide. In previous studies (see Chapter II), involving reproductive implants in cobia (*Rachycentron canadum*), dermal and musculature lesions and secondary infections have resulted after injection with Ovaplant® implants using the Ralgun® implanter.

In previous reproductive studies, lesions caused by the implanting process have resulted in mortality of adult cobia held in recirculating aquaculture systems at the Texas A&M University Aquacultural Research and Teaching Facility. Small lesions were observed 2–4 days post-implantation which increased to larger, deep ulcerations 5–10 days post-implantation and the infected broodstock were euthanized 25 days post-

implantation after trial criteria for euthanasia was reached and recovery was deemed unlikely to occur by research personnel. Negative side effects of the injection of a similar hormone, Ovaprim®, were observed by Hill et al. (2009) and Lipscomb et al. (2018); these adverse effects included redness and bruising at the injection site and stemmed from degradation of water quality while holding and handling fish and in some cases led to mortality.

Another issue identified from previous studies is a complication related to the fixed dose of each implant (Hill et al. 2009, Sink et al. 2010, DiMaggio et al. 2014, Lipscomb et al. 2018). There is no way to ensure fish receive the correct biologically active dosage based on body weight because each implant contains a static dose with large intervals among available implant doses (Sink et al. 2010). Small fish can receive an overdose when using even the smallest commercially available implant dosage (Sink et al. 2010). Large marine fish can be implanted with multiple implants to get close to the desired biological concentration, but it is difficult to achieve the correct body weight dosage without using multiple implants and implant doses. Hormone implants are extremely costly compared to hormone injections, often costing more than USD$10 per implant, and can only be purchased commercially in implant cartridges containing 24 implants (Sink et al. 2010). To purchase and keep on hand multiple implant cartridges, each containing implants of different doses to achieve dosages close to desired biological concentrations, adds a great deal of expense when considering they only have a one-year shelf-life and only 5–10 broodfish may be implanted in any given year to supply an entire farm. Even then, the applicator can only achieve a dosage close to the

desired biological concentration. Due to this fact, broodfish often receive too much or too little of the hormone when injected with the implant (Sink et al. 2010). These observations led other researchers and myself to conclude that a less invasive procedure that can deliver exact biological dosages of peptides based upon fish weight is needed to induce spawning in large marine fish.

Other animal production industries, such as beef and swine production, use liquid- or gel-based excipients to deliver and slowly release peptides over an extended period of time. Examples of these are slow-release bovine somatotropin (bST, Prosilc, Elanco, Greenfield, Indiana) to induce and promote lactation in cows (Gadeken and Casper 2017) and LONGRANGE® (eprinomectin 5% w/v extended-release injection, Boehringer Ingelheim Animal Health USA Inc., Duluth, Georgia) to prevent bovine warble fly infections (Lia et al. 2019). These excipients could potentially be adapted for use in fish, allowing for customizable peptide doses to be drawn into a syringe and delivered to the fish intramuscularly through needles as small as 23 gauge. These excipients would still allow the same time-release benefit as implants do, in a customized dose for each fish that is less intrusive. In large marine broodstock, this also would result in fewer puncture wounds, one for injection versus multiple for implants, created in the dermis and musculature of the fish. The resulting puncture wound also would be significantly smaller than the 4.7625-mm puncture wound created by the Ralgun® implanter used to administer Ovaplant® implants. This would reduce the exposure of the fish to exogenous bacterial and fungal pathogens in the aquatic environment by limiting wound sites and size where pathogens could enter.

One such excipient used in beef and swine production that has potential to be used in fish is a hydrophobic, sucrose-based liquid with a propriety blend of emulsifiers that slowly releases peptides over an extended period. (This excipient is under an Non-disclosure agreement). The majority (~60%) of the peptide is released from the excipient within 24 h of injection while the remaining 40% of the peptide is slowly released from 24 to 72 hours post-injection (Dr. Peter McKenzie, Senior VP Product Management, and Dr. Katie Haman, DVM, Director for Spawn Products, Syndel, Ferndale, Washington). This is somewhat analogous to a priming dose and later resolving dose of hormones frequently employed with fish when using aqueous-based peptide injections, but eliminates the need to repeatedly capture, handle, inject, and otherwise stress the fish. Additionally, it delivers a steady flow of peptide for 72 hours post-injection unlike a static resolving dose that delivers the peptide to the fish all at once. This is a novel excipient and it has never been used on any species of fish prior to this clinical trial to make observations of gross pathology of the excipient.

The first step in evaluating the safety and efficacy of an excipient is to determine the safety and conversely any adverse effects when administered to the test animal. In order to establish the effects on the test animal, a "placebo" injection, or simply an injection containing only the excipient and no peptide, must be evaluated in the test animal to determine the effects of only the excipient on the animal. Red drum (*Sciaenops ocellatus*) have been spawned in captivity since the 1970's (Davis 1990) using either spawning peptides or environmental conditioning, and they are the most readily available species of warm-water marine fish cultured in the United States. Therefore,

they are an ideal test subject to evaluate the effects of a sucrose-based excipient in warm-water marine fish.

Objectives and Hypotheses

The primary objective of this clinical trial was to assess the safety and conversely any adverse effects of a sucrose-based excipient when injected into the dorsal musculature and or intra-coelomic cavity (IC) of red drum, specifically investigating if the placebo causes a reaction and/or pathology at the site of injection. The research hypothesis was that red drum will show signs of gross pathology and reaction upon injection with placebo replacement of hormones.

Methods and Procedures

Red drum (n = 20) were kept in a 4,429-L recirculating tank, at a photoperiod of 14 h of light and 10 h of dark at a water temperature of 24–26° C and a salinity of >10ppt.. The system included a 2m x 0.5m-sump tank filled with two sacks of bioballs/filtration media for water collection, a 2-horsepower pump (Hayward MaxFlo XL 2 HP Dual Speed Pool Pump, One Hayward Industrial Drive Clemmons, North Carolina), a Arias 4000© (Pentair Aquatic Eco-Systems Inc. 2395 Apopka Blvd. Apopka, Florida) bead filter, a 25-micron Water Co.© filter (Water Co.®, Augusta, Georgia), and a Jebao© PU-36 UV clarifier (Jebao®, Dongsheng, Zhongsha, Guangdong, China). One half of the water that exited the UV filter was returned to the sump tank. The other half of the water that exited the UV filter returned to the tank at an angle encouraging uni-directional waterflow in a circular pattern. The tanks were set up

with a venturi drain style standpipe, covered in large mesh that let waste water flow into a drainage pipe that deposited waste water into the sump tank for filtration. Aeration was applied using four air stones from an external blower (Whitewater® Regenerative Blower Pentair Aquatic Eco-Systems Inc. 2395 Apopka Blvd. Apopka, Florida).

Twenty-five milliliters of a proprietary sucrose-based excipient placebo in a sealed syringe vial was supplied by Syndel International Inc. The excipient was stored under refrigeration (2–8°C) until time of the clinical trial to ensure effectiveness and limit potential expiration. Approximately 45 minutes prior to use, the excipient was removed from refrigeration and allowed to warm to room temperature (23°C) to ensure viscosity and ease of drawing the excipient into the syringe**.** Following removal of the placebo from refrigeration, if liquid was still too viscous to draw into a 21-gauge needle (BD PrecisionGlide™ Needle, 21G x 2 [0.8mm x 50mm], Becton, Dickinson and Company, 1 Becton Drive Franklin Lakes, New Jersey), it was warmed by hand for 5 minutes to decrease viscosity.

Red drum were removed from the holding tank, anaesthetized using 70 mg/L sodium bicarbonate buffered tricaine methanesulfonate (Western Chemical Incorporated, Ferndale, Washington) and a mixture of Vidalife™ (Syndel International Inc., Qualicum Beach, British Columbia, Canada) and sea water in a holding bath for 2 minutes with oxygen provided from a 6.23-cubic meter compressed-oxygen cylinder at a rate of 2-L/minute using a fine pore, porcelain air stone (69.9 cm x 8.5 cm, Point Four™ Micro Bubble Diffusers, Pentair Aquatic Eco-Systems, Inc., Apopka, Florida) and a regulator

(Roscoe Medical, RMI-15H CGA-540 H Regulator, 0-9 LPM, Compass Health Brands, 6753 Engle Road Middleburg Heights, Ohio).

Fish were administered 0.1 mL of the excipient by injection into the right dorsal musculature between the first and second dorsal fins approximately 2.5 cm below where the dorsal fin meets the body musculature. A second 0.1 mL injection of the excipient was administered to the intra-coelomic cavity (IC), approximately 5 cm anterior and to the left side of the urogenital pore. Excipient injections were administered using sterile 3-mL syringes (Becton, Dickinson and Company, Luerlok disposable syringes,1 Becton Drive Franklin Lakes, New Jersey) and 21-gage needles (BD PrecisionGlide™ Needle, 21G x 2 [0.8 mm x 50 mm]) . A new syringe and needle were used for each fish and injection site to minimize infection risks. After injection fish were released back into the holding tank.

Fish were monitored for mortality and fed their normal diet of Rangen EXTR 400™ (Rangen Inc., Angleton, Texas) 0.32 cm pelleted feed for 8 days post-injection. Eight days days (3 days of hormone delivery followed by a 5-day latency period) was determined to be the end point of the clinical trial because had the excipient contained an actual spawning peptide, all viable broodfish receiving the excipient would have spawned within this period. If mortality occurred, the specimen was immediately refrigerated (2–8°C) until a post mortem exam/necropsy could be performed (<12 hours).

Analysis

At the conclusion of the 8-day clinical trial, all specimens were euthanized by removing them with a net from the holding tank and placing them in a 1,000 L-tank filled with a mixture of seawater and an overdose of isoeugenol (Sigma-Aldrich® PO Box 14508 St. Louis, Missouri) 150 mg/L. Following euthanasia, post-mortem exams/necropsies were conducted. During post-mortem exams, injection sites were first visually observed externally and any abnormalities, lesions, etc. were noted. Next, the scales and dermis covering the injection sites were removed with a scalpel (Disposable safety scalpel 23, Integra LifeSciences, 311 Enterprise Drive, Painsboro, New Jersey) and the fish was inspected between the dermis and outer musculature for signs of pathology including needle punctures, discoloration, hemorrhaging, inflammation, lesions, pustules, nodules, infection, or scar tissue. When any signs of pathology were found, they were recorded and examined microscopically (T720 Amscope, 40X-1000X Plan Infinity Kohler Laboratory Trinocular Compound Microscope, Irvine, California). The musculature was then sliced layer by layer using a scalpel to investigate for evidence of pathology, as listed above, associated with the injection site or excipient.

After inspection and dissection of the dorsal musculature, the IC injection was then inspected by using a scalpel to open a 6–8 cm incision beginning at the urogenital pore towards the anterior of the fish. The incision was spread open using tweezers and inspected for signs of the excipient or pathology including needle punctures, discoloration, hemorrhaging, inflammation, lesions, pustules, nodules, infection, or scar tissue. The organs around the injection site also were evaluated for reaction to the

injection. When any signs of pathology were found, they were recorded and examined microscopically. If gross pathology was observed in a specimen, small pieces of tissue were collected and stored in formalin prior to histopathology. All results of post-mortem exam/necropsies were recorded for later analysis.

Observations

Survival of red drum after treatment was 100% ± 0 (SE) and no signs of primary or secondary bacterial infections were noted in or around the injection sites, and all fish appeared healthy. Only 5 (25%) of the 20 fish injected showed effects of injections. Two (10%) of the fish were observed with a small incision in muscle tissue caused by a needle during injection. One (5%) fish had a small nodule of scar tissue in proximity of an injection site and 2 (10%) fish had subdermal inflammations observed at the dorsal injection site.

No lesions or ulcerations were found around the injection sites. Minimal effects of the injections were noted such as scar tissue, puncture wounds, hemorrhaging, discolored livers and globules of the excipient located under the dermis (Fig. 3.1). In this clinical trial, normal behavior of the fish resumed in about an hour and the fish ate vigorously only 3 hours post-injection. For 33% of the IC injections, a small discolored dot on the outer dermis was noted , where the injection was administered (Fig. 3.2). Observations of the dorsal IM injection site found one fish (5%) had a small incision in interior muscle tissue (Fig. 3.3) probably caused by dorsal injection site (Fig. 3.4). There was globular, tacky, gelatinous substance (Fig. 3.5)

Figure 3.1. In 75% of the fish, the IC injection was completely unremarkable and no adverse effects at the injection site were noted.

Figure 3.2. As observed in this fish, a small discolored dot on the outer dermis where the IC injection was administered was noted on 33% of the fish in the clinical trial.

Figure 3.3. A small incision in the interior muscle tissue as noted in this fish was caused by the needle during injection.

Figure 3.4. Note the subdermal inflammation observed at a dorsal injection site of this fish.

Figure 3.5. Globular, tacky, gelatinous substance believed to be part of the injected hormone excipient that was forced out of the muscle tissue at the injection site of this fish.

removed from subdermal pocket (Fig. 3.6). It was believed to be part of the injected hormone excipient that was forced out of the muscle tissue at the injection sight. It was noted during injection that some of the initial injections were very shallow in the dorsal muscle tissue. It was observed the hydrophobic, sucrose-based liquid excipient with a proprietary blend of emulsifiers, became a sticky, gelatinous globule once injected into the fish. On a fourth fish, an incision was observed in muscle tissue made from needle during injection; however, there was no bleeding, bruising or scar tissue noted. In a fifth fish, a small nodule of scar tissue was observed in muscle tissue in proximity of the

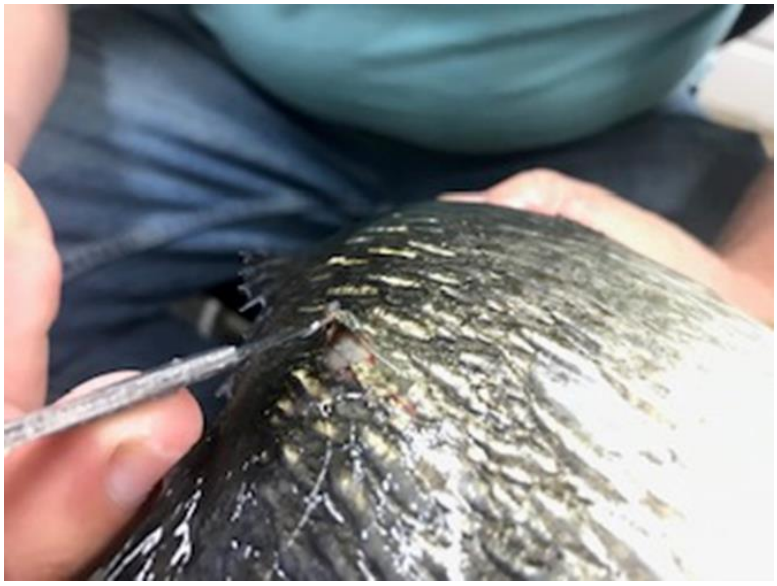


Figure 3.6. A subdermal pocket located at dorsal IM injection site of this fish wherein a tacky, gelatinous substance was observed in this fish.

Figure 3.7. One of 15 (75%) fish where no notable abnormalities were observed at the IM injection site.

injection site. The scar tissue was not immediately discernable as a result of the excipient or injection because of the healed status of the scar tissue in relation to the time of injection to the time of dissection. The cause was not likely due to the injection or excipient. No abnormalities were observed for the other 15 fish injected (Figs. 3.1 and 3.7).

Discussion

When compared to traditional spawning aids, this sucrose-based excipient was less invasive, easier to tailor dosage, and caused minimal harm and discomfort to the fish. It is important to note that this is the first exploratory clinical trial of this excipient and there is no previous research on the effects of this excipient.

Other spawning aids such as Ovaplant® and Ovaprim® also have led to secondary bacterial infections, lesions, and mortality (Sink et al. 2010, Hill et al. 2009). Hill et al. (2009) also found that negative effects of Ovaprim® injection included redness and bruising at the injection site and stemmed from degradation of water quality while holding and handling this fish. The time release action of this injection mitigates the prolonged or multiple handlings of this fish which reduces stress and potential mortalities.

Due to the novel nature of this excipient, I reviewed the actual use, protocols, and observations based on the clinical trial. The excipient used in this research was extremely viscous. I allowed the excipient to sit at room temperature (26.7 C) for 25 minutes and it was still difficult to draw into a syringe. I allowed the excipient to remain at room temperature for another 15 minutes before drawing it into the syringe; however,

it still required approximately 1 minute to draw a 0.1-mL dose of excipient into the syringe using 23-gauge needles. This was not practical with 40 syringes to fill and would not be acceptable in a commercial setting where hundreds of fish may need to be injected in a source period of time. Therefore, I switched to 21-gauge needles, but the excipient was still somewhat difficult to draw into the syringe. Using 21-gauge needles was more practical and required approximately 15–20 seconds to draw the 0.1-mL dose of excipient into the syringe.

Despite the difficulties of drawing the excipient into the syringe, the excipient was not difficult to inject into the fish. However, the viscosity issue can prove problematic for both large broodfish such as cobia and small broodfish such as ornamentals. For large broodfish where larger volumes or excipient and hormone are required, even a 21-gauge needle may prove impractical to quickly load large volumes and larger needles would be required. Larger needles would of course leave larger holes which could lead to a higher secondary infection rate. For small ornamental fish, the use of a 21-gauge needle or larger could be damaging to the fish.

In all, the viscosity could be an issue, but this excipient would be an improvement over the large and numerous holes in the dermis created by the Ralgun® implanter that are required to put 6–8 implants into a single large broodfish to achieve the correct dosage. However, it could provide a slow-release option for small broodfish where the large cellulose-based implants are impractical and potentially lethal. From my experience with the excipient, it appears to be quite safe and minimally invasive for the

fish. As noted, once the excipient was inside the fish, it formed a sticky, gelatinous, globule that did not appear to cause distress or physical damage to the fish.

While this was the first clinical trial using this sucrose-based excipient, it has the potential to replace commonly used hormone implants or injections. It was non-invasive and due to its time-release properties, it limits handling time that may cause undue stress to fish. The positive implications of the results of this clinical trial indicated this excipient could be used as a spawning aid for most species of fish with no or very few adverse effects.

CHAPTER IV

OVERCOMING MALE SKEWED GENDER BIAS DUE TO TEMPERATURE DEPENDENT GENDER DETERMINATION IN SOUTHERN FLOUNDER (*Paralichthys lethostigma*) BY EVALUATING METHODS TO CREATE GYNOGENTICAL, CLONED PROGENY

Introduction

Southern flounder (*Paralichthys lethostigma*) is an important sport and commercial foodfish species. It tolerates a wide range of salinities and temperatures which make it a prime candidate for intensive aquaculture (Daniels and Watanabe 2003, Watanabe et al. 2006). Southern flounder has an established market and there is interest in improving aquaculture techniques for this species. Advances in culture have been made, including successful spawning of broodstock collected from the wild, larviculture, and grow out (Watanabe et al. 2006). However, these successes have been limited. Large-scale culture as food fish or production in quantities necessary to supplement wild populations through stock enhancement programs has not been realized (Copeland et al. 1998).

Reliance upon wild broodstock is due to two primary reasons. The first is that milt has been difficult to obtain reliably in captive-held broodstock, even when spawning peptides are used. Males must be checked regularly for milt production by "stripping" them through application of pressure to the posterior sides of the abdomen, and maintaining pressure while stroking forward along the abdomen toward the anterior

portion near the urogenital pore. This process induces stress, can damage internal organs, and removes the mucus coat and scales potentially subjecting the fish to a host of secondary bacterial pathogens in the aquatic environment. This process results in a high incidence of disease and mortality in male broodstock (Paul Cason, Hatchery Manager, Texas Parks and Wildlife Department, personal communication).

The second reason is males are often quite small relative to the much larger, sexually-mature females (Fitzhugh et al. 1996, Monaghan and Armstrong 2000, King et al. 2001). The two genders cannot be maintained together in tanks or ponds after spawning as females frequently attack smaller males in an apparent attempt to consume them. This is attributed to an attempt to quickly recover energy lost from egg production and spawning. Thus, females may be used for several years, but unless each spent female can be removed from spawning tanks immediately after spawning, at least a portion of males may be killed or receive wounds resulting in secondary infections and frequently mortality. For these reasons, a portion to all of the males required for spawning must be replaced by capturing new broodstock from the wild each year. Once culture of this species becomes more refined, it may be possible to produce sufficient quantities of males needed each year for production.

Also problematic is the skewed gender ratio that can result when a species is cultured in an unnatural or disturbed environment (e.g., environmental temperature during larval and juvenile stages can affect sex differentiation in fish). Gender can be determined by a single variable such as genetics or by multiple variables (Nakamura et al. 1998, Baroiller et al. 1999, Pandian and Koteeswaran 1999). Flounder species exhibit

differentiation in gender due to genetics, water temperature during juvenile development, and circulating levels of stress and sexual hormones, with temperature being the strongest influencing factor (Luckenbach et al. 2003). Southern flounder females are homogametic (XX) and males are heterogametic (XY; Luckenbach et al. 2004). During development, high (>25°C) and low (<22°C) temperatures result in more (51–100%) phenotypic, functional males (Yamamoto 1995, 1999). Gender determination in southern flounder occurs between 75 and 120 mm total length (Luckenbach et al. 2003). A rearing temperature of 23°C during the period of sexual differentiation promotes more phenotypic females and a gender ratio close to 1:1 (Luckenbach et al. 2003), but this condition is difficult to maintain in a large-scale hatchery facility where water temperatures are not easily controlled. During early sexual differentiation, homogametic individuals can be forced to differentiate as phenotypic males through exposure to high or low water temperatures (Luckenbach et al. 2003) or exogenous androgens such as 17α-methlytestosterone, resulting in genetic females that are phenotypic, functioning males (Luckenbach et al. 2004).

Monosex population production of other fish species is often used to prevent unwanted production of one gender due to inferior traits or to delay sexual maturation and prevent the redirection of nutrients from growth to reproduction (Donaldson 1996). Male southern flounder rarely achieve marketable size (>600 g), even after three or more years of culture. Monosex culture of genetically and phenotypically all female flounder could alleviate the issues that arise with sexual dimorphism and the skewed gender ratios resulting from nonoptimal rearing temperatures.

The aforementioned bottleneck has been addressed in previous trials that have applied diploid genesis in similar species such as plaice (*Plueronectes platessa*) and European flounder (*Platichthys flesus*; Purdom 1969), and in southern flounder using UV irradiation of sperm followed by either a cold or pressure shock of the activated eggs (Luckenbach et al. 2004, Morgan et al. 2006). Luckenbach et al. (2004) found the induction of diploid genesis using cold-water shock was successful in production of gynogenetic diploids. Morgan et al. (2006) tested the ability of a pressure shock to retain the second polar body with limited successes.

These experimental methods to resolve the male gender bias created by temperature-regulated sexual differentiation have never been evaluated concurrently to determine the most effective treatment. Diploid genesis is a two-part process. First gynogenesis is induced through egg activation with inactivated spermatozoa and secondly expulsion of the second polar body is blocked through a shock treatment (Luckenbach et al. 2004). Inactivated sperm created through an irradiation process to render the genetic material non-viable serves to trigger completion of meiosis in activated eggs, but does not contribute genetically to the offspring. When sperm is inactivated and expulsion of the second polar body is prevented by a shock treatment applied to activated eggs, the gynogenetic diploid, or gynogen, inherits both chromosome sets from the female parent (Luckenbach et al. 2004). This process creates progeny that are gynogenetic clones, possessing the same chromosomes, DNA, and mitochondrial RNA as the mother. These genetically all female offspring, or gynogens, can then be subjected to temperatures greater than 25°C during juvenile development or

exogenous androgens like 17α-methlytestosterone resulting in phentotypic, functional males, or super XX males. Super XX males can be reared to maturity and bred back to genetically and phenotypically normal females to produce progeny that are genetically all female (Fig. 4.1; Yamamoto 1995).

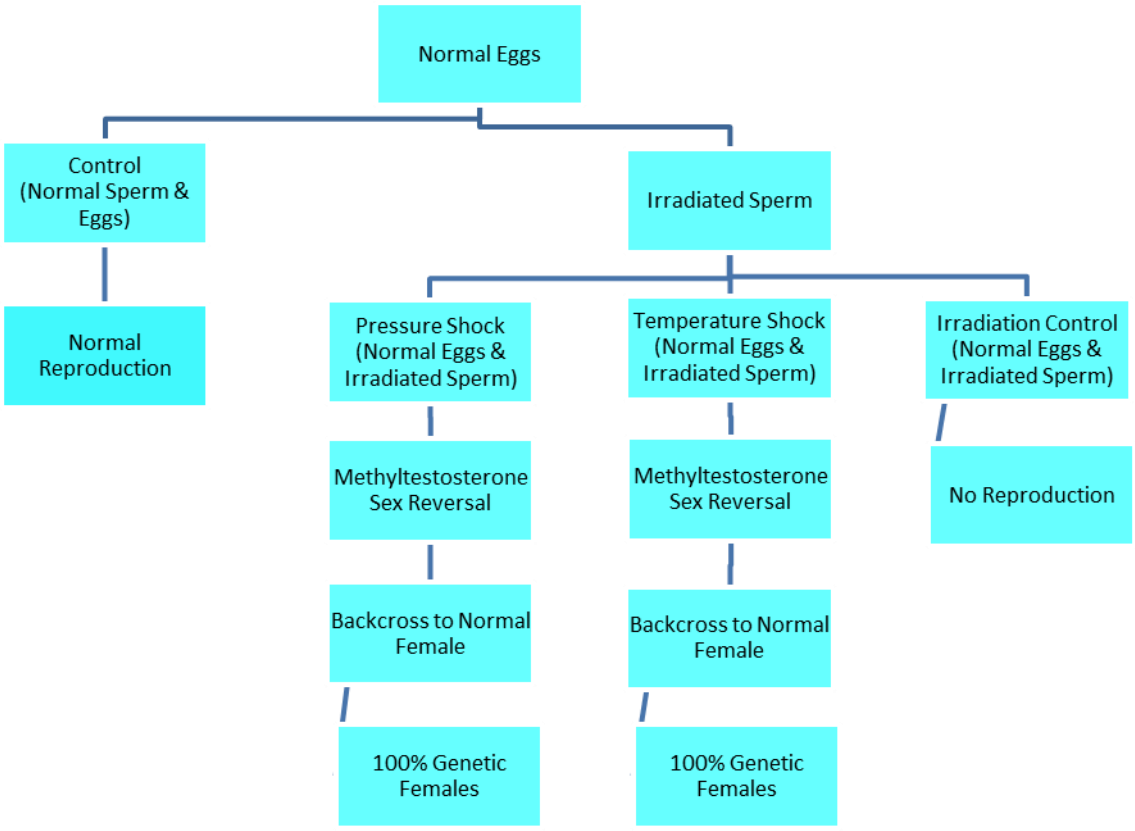


Figure 4.1. The process by which gynogenetic flounder juveniles can be sex reversed after cold- or pressure-shock treatments and used as broodfish to create all female flounder populations.

Objectives and Hypotheses

The primary objective of my research was to determine which shock treatment (pressure or cold shock) to induce diploid genesis produces more viable offspring in southern flounder. The research hypothesis was that pressure and cold shock treatments will produce a similar number of viable offspring.

Methods and Procedures

Broodstock Acquisition and Transport

Flounder broodstock (n = 8) were obtained from Sea Center Texas in Lake Jackson, Texas and transported in a 1,325-L insulated hauling tank to the Aquacultural Research and Teaching Facility (ARTF; 13950 FM 60 East, Somerville, Texas). Sea Center Texas participates in a state-sponsored southern flounder stock enhancement program and supplied eight adult fish that were on a 150-day photoperiod/temperature cycle to spawn from their broodstock for this research.

The insulated hauling tank was filled with 30 g/L seawater at 25°C prior to transport. The hauling tank was supplied with supplemental oxygen from a 6.23 cubic meter compressed oxygen cylinder and dissolved oxygen (DO) at 2-L/minute (Regulator; Roscoe Medical, RMI-15H CGA-540 H Regulator, 0-9 LPM Compass Health Brands, Middleburg Heights, Ohio) and fine pore, porcelain air stones (69.9 x 8.5 cm, Point Four™ Micro Bubble Diffusers, Pentair Aquatic Eco-Systems, Inc., Apopka, Florida). Vitalife® (Syndel International Inc., Qualicum Beach, British Columbia, Canada) slime coat was added to the tank water to preserve the mucus layer

of the fish and prevent slime accumulation on the water surface that could potentially limit oxygen exchange. These procedures were intended to limit stress on fish.

Broodstock Acclimatization, Care, and Feeding

Broodstock were kept in a 3.65-m diameter, 0.76-m deep tank operated as a recirculating aquaculture system. Broodfish were environmentally conditioned using established protocols for salinity, photoperiod, and temperature (Smith et al. 1999, Watanabe et al. 2001). These protocols were: temperature 19 ± 1°C, salinity 30 g/L, 12 h light:12 h dark, and D.O. ≥6.0 mg/L. Fish were fed twice daily with frozen shrimp trawl bycatch including croaker, squid, and shrimp and 10 g of prepared Mazuri® Spartan Aquarium Gel Diet (Mazuri Exotic Animal Nutrition, St. Louis, Missouri).

Hormone Injection, Milt Collection, and Sperm Count

Males were removed from the holding tanks, anaesthetized using 70 mg/L sodium bicarbonate buffered tricaine methanesulfonate (Western Chemical Incorporated, Ferndale, Washington) and a mixture of Vidalife™ (Syndel International Inc., Qualicum Beach, British Columbia, Canada) and sea water in a 210-L anesthesia bath for 4 minutes. Oxygen was provided from a 6.23-cubic meter compressed oxygen cylinder at a rate of 2-L/minute using fine pore, porcelain air stone. Weight was recorded and males (n = 3) were administered 0.5 mL/kg body weight of Ovaprim® (Syndel International Inc., Qualicum Beach, British Columbia, Canada) via intramuscular (IM) injection in the dorsal musculature below the dorsal fin using sterile 3-mL syringes (Becton, Dickinson and Company, Luerlok disposable syringes, Franklin Lakes, New Jersey) and 21-gage

needles (BD PrecisionGlide™ Needle, 21G x 2 [0.8mm x 50mm], Becton, Dickinson and Company, Franklin Lakes, New Jersey) to exacerbate milt production. A new syringe and needle were used for each fish to minimize infection risks. After injection fish were released back into the culture tank to recover.

Thirty to 48 hours post-injection, milt was collected into a 2-mL cryovial and sperm count and motility was recorded for each male. Sperm counts were performed by adding 5 µm of sperm and 250 µm of Ringer's solution (0.75% NaCl, 0.04% $CaCl_2$, 0.02% KCl in distilled water; Luckenbach et al. 2004, Morgan et al. 2006) into a 5-mL cryovial and subjected to vortex mixing at 240 rpm for 5 seconds. After mixing, 5 µm of the milt and Ringer's solution mixture were placed on a counting slide, 10 µm of artificial seawater (35 g/L salinity; Red Sea Salt, Red Sea USA, Houston, Texas) were added to activate the sperm, and total and activated sperm was counted per grid microscopically at 100x magnification. Sperm counts and motility were recorded from each individual male, and equal portions of milt from 2 to 3 males was combined and vortex mixing at 240 rpm for 5 seconds in a 2 mL cryovial. That mixture was then divided into two samples and diluted 1:10 with Ringer's solution. Half of the sperm was placed into a 2-mL cryovial and kept in an ice bath for use as a control. The other half of the sperm was spread thinly onto a 70-mm glass petri dish and irradiated for 40 seconds at 70 J/cm^3 using a Fisherbrand™ UV crosslinker with adjustable height (13-245-221; Fisher Scientific, Pittsburg, Pennsylvania) according to methodology from Morgan et al. (2006). The irradiated sperm was then placed into a 2-mL cryovial and kept in an ice bath until use.

Female Hormone Injection and Egg Collection

Females were removed from the holding tank and anaesthetized using 70 mg/L of MS-222 and a mixture of Vidalife™ and sea water in a 210-L holding bath for 4 minutes. Oxygen was provided from a 6.23-cubic meter compressed oxygen cylinder at a rate of 2-L/minute using fine pore, porcelain air stone. Weight was recorded for each female. Females were placed onto a light table to assess gonadal development. Stages of gonadal development were identified on a scale from 1–4 (Daniels and Watanabe 2003). Stage 1 indicated little discernable development, stage 2 indicated discernable development and slight abdominal swelling, but gonads only to less than halfway back to the caudal fin, stage 3 indicated abdominal swelling and gonadal extension greater than three quarters of the way back to the caudal fin, and stage 4 indicated gonads were fully extended to the caudal fin as well as pronounced abdominal swelling and identification of a small clear area near the oviduct (Daniels and Watanabe 2003). Only females exhibiting stage 3 or 4 were to be chosen for injection, and all five females met this criterion. Females were injected IM with 0.5 mL/kg body weight of Ovaprim® to induce egg maturation and ovulation. Females were kept in individual 113-L holding tanks separate from males during the period of latency following hormone injection.

Starting 30 h post-hormone injection and every 4 h thereafter, ovarian maturation stage was again assessed using the light table and the fish were examined for ovulation. To determine if ovulation has occurred, females were placed upon a foam pad and gentle pressure was applied to the abdomen with an anterior stroking motion in an attempt to "strip" ovulated eggs from the female. When ovulation was determined to have

occurred, the females were strip spawned using techniques defined by Rottmann et al. (1991) and the eggs were collected in a dry glass beaker. The quantity of eggs collected from each female was recorded by placing the eggs into a 1,000 mL graduated cylinder filled with seawater and enumerating the number of eggs per 1-mL sample. After eggs were collected, broodfish were placed in a holding tank with a high rate of aeration to recover. The eggs from each female were divided into four, 70-mm glass petri dishes, one for each of two controls and two treatments.

Non-irradiation Control

A non-irradiation control consisted of eggs fertilized with non-treated milt, and served to determine egg viability and fertilization rate of normal spawns. Milt, (250 μL) was added to the eggs within a petri dish, the mixture was gently swirled for 60 seconds to distribute the milt throughout the eggs, and 40 mL of artificial seawater (35 g/L salinity) was added to activate the sperm and initiate fertilization. The dish was then gently swirled for 3 minutes following the addition of seawater. Fertilized eggs were placed into replicate (n = 6) incubation tanks with light aeration to keep the eggs gently "rolling" and 50% water exchange daily (Daniels et al. 1996). The quantity of fertilized eggs, quantity of non-fertilized eggs, and fertilization percentage was recorded at 24 h post-fertilization, and percentage of eggs with developing embryos was recorded at 36 to 40 h post-fertilization.

Irradiation Control

The second control, irradiation control, consisted of eggs activated using

irradiated sperm, but received no shock treatment to retain the second polar body. Irradiated sperm plasma fuses with the egg's plasma membrane and merely activates the egg to start development and undergo cellular division. True fertilization does not take place as there is as no transfer of genetic material from the male. Fertilization only takes place when the nucleus of both a sperm and an egg fuse to form a diploid cell, known as zygote. This negative control determined the effectiveness of the irradiation treatment to render the genetic material contained within the sperm non-viable. Irradiated milt, approximately 250 μL, was added to the eggs within a petri dish, the mixture was gently swirled for 60 seconds to distribute the milt throughout the eggs, and 40 mL of artificial seawater (35 g/L salinity) was added to activate the sperm and initiate egg activation. The dish was then gently swirled for 3 minutes following the addition of seawater. Activated eggs were placed into a 2,000-ml glass beaker containing 1,500 mL seawater (35 g/L salinity) with light aeration for incubation. The quantity of activated eggs, quantity of non-activated eggs, and activation percentage was recorded at 24 h post-milt addition, and percentage of eggs with developing embryos was recorded at 36 to 40 h post-milt addition.

Chromosomal Manipulation Using UV-Irradiated Sperm

Cold-Shock Treatment

The third petri dish of eggs from each female was activated using irradiated sperm prior to being subjected to a cold-shock to induce diploid gynogenesis by preventing expulsion of the second polar body. Polar bodies serve to eliminate one half

of the diploid chromosome set produced by meiotic division in the egg, leaving behind a haploid cell. When expulsion or chemical disintegration of the second polar body is prevented, both sets of the maternal chromosomes remain within the egg creating a diploid cell containing only genetic material from the mother (Chourrount 1984).

Irradiated milt (250 μL) was added to the eggs within a petri dish, the mixture was gently swirled for 60 seconds to distribute the milt throughout the eggs, and 40 mL of artificial seawater (35 g/L salinity) was added to activate the sperm and initiate egg activation. The dish was then gently swirled for 3 minutes following the addition of seawater. The eggs were gently poured into a 500-mL clear acrylic cylinder with a 250-μm mesh bottom to allow water circulation. The cylinder and eggs were submerged into a circulating water bath (Isotemp 3013 circulating chiller water bath, Fisher Scientific, Pittsburg, Pennsylvania) at 0°C for 45 minutes (Luckenbach et al. 2004).

After 45 minutes, the water bath containing the cylinder and eggs was quickly heated to 18°C during a 12–15-minute period. Cold-shocked, activated eggs were placed into replicate incubation tanks (n = 6) with light aeration to keep the eggs gently "rolling" and 50% water exchange daily (Daniels et al. 1996). The quantity of activated eggs, quantity of non-activated eggs, and activation percentage were recorded at 24 h post-milt addition, and the percentage of eggs with developing embryos were recorded at 36 to 40 h post-activation.

Pressure-Shock Treatment

The fourth petri dish with eggs from each female were activated using irradiated

sperm prior to being subjected to a pressure-shock to induce diploid gynogenesis by preventing expulsion of the second polar body. Irradiated milt (250 μL) was added to the eggs within the petri dish, the mixture was gently swirled for 60 seconds to distribute the milt throughout the eggs, and 40 mL of artificial seawater (35 g/L salinity) was added to activate the sperm and initiate egg activation. The dish was then gently swirled for 3 minutes following the addition of seawater. The eggs were then gently transferred into a hydrostatic pressure chamber (Aquatic Ecosystems, Apopka, Florida) filled with sea water. The hydrostatic pressure chamber was immediately pressurized to 8,500 psi (pounds/square inch) of pressure for 6 minutes before releasing the pressure (Morgan et al. 2006). Pressure-shocked, activated eggs were placed into replicate incubation tanks (n = 6) with light aeration to keep the eggs gently "rolling" and 50% water exchange daily (Daniels et al. 1996). The quantity of activated eggs, quantity of non-activated eggs, and egg activation percentage were recorded at 24 h post-milt addition, and percentage of eggs with developing embryos were recorded at 36 to 40 h post-activation.

Larval Rearing

Controls and treatments were replicated six times each using four individual spawns from different females and two spawns from a single female. The incubation system included a 1 m x 0.5 m sump tank for water collection, a 1 horsepower pump (Hayward® MaxFlo 1 HP Dual Speed Pool Pump), a sand filter (Arias 4000© Pentair Aquatic Eco-Systems Inc., Apopka, Florida), a canister filter (Water Co.©, Augusta, Georgia) with a 5 μm paper filter, and a 36-watt UV filter (a Jebao© PU-36 UV clarifier

(Jebao®, Dongsheng, Zhongsha, Guangdong, China). The tanks were set up with a slotted internal standpipe covered with 250-µm nylon mesh that led to an external standpipe that maintained water level. Temperature was controlled by a chiller/heater (Aqualogic®, San Diego, California). Temperature was maintained at 18 ± 2°C, salinity maintained at 30 g/L, and DO was maintained at greater than 6 mg/L. Incubation tanks were subjected to a 12-hour light and 12-hour dark cycle. Aeration was maintained at a low level for the duration of the experiment to prevent damage to larvae from excessive water turbulence. All experimental methods and culture practices were conducted under Texas A&M University Animal Use Protocol: 2016-0279.

Larval Feeding

One hundred milliliters of live *Nannochloropsis* spp marine microalgae (Reed Mariculture Inc., Campbell, California) were added to each larval tank daily starting day 1 post-hatch for 17 days. Starting at day 2 post-hatch, larval fish were fed rotifers enriched with N-Rich PL Plus® (Reed Mariculture Inc., Campbell, California) at a concentration of 15 rotifers/mL of tank water twice daily through day 19 post-hatch (Daniels and Hodson 1999). Starting day 6 post-hatch, larval fish were offered 1 g of dry diet (Otohime diet A1, Reed Mariculture Inc., Campbell, California) three times daily through day 22 post-hatch (Daniels and Hodson 1999). Starting day 13 post-hatch, larval fish were fed 32,500 *Artemia* nauplii (Great Salt Lake strain, INVE AQUACULTURE INC., Salt Lake City, Utah) per tank twice daily. *Artemia* nauplii were fed through day 37 post-hatch (Daniels and Hodson 1999). Incubation tanks were set for a 12-hour light and 12-hour dark cycle schedule. Gentle aeration was maintained for the duration of the

experiment. Water exchange was increased to 100% exchange per day at 3 days post-hatch. Tanks were siphoned by hand to remove dead eggs, egg chorions, and waste every other day starting 4 days post-hatch until 45 days post-hatch (Daniels and Hodson 1999).

Statistical Analyses

The IBM SPSS® software platform (IBM, Armonk, New York) was used for all analyses. A statistical power test was conducted using the software platform to determine minimum sample size necessary to reject the null hypothesis when the null hypothesis is false, while using the minimum number of animals to accomplish the study for all experimental methods stated above. Data for each trial were analyzed using a one-way analysis of variance (ANOVA). A $P \leq 0.05$ was taken to indicate statistical significance among treatment means. When significant differences among means were found, treatment means were separated using Duncan's multiple-range post-hoc test. The alpha for the power analysis was set at 0.05 and the power of the test was set at 0.90 to generate the sample size. In addition, a General Linear Model was performed to model the relationship between percent egg fertilization/activation, percent embryo production, and treatment.

Results

There was a significant ($F = 9.93$, $df = 2$, $P = <0.0001$) difference between the mean egg fertilization/activation rates of the irradiated control, non-irradiated control, irradiated pressure-shock treatment, and irradiated cold-shock treatments. However, there was no significant difference between the non-irradiated control, irradiated

pressure-shock treatment, and irradiated cold-shock treatment. Between 21 and 22.5 hours post-fertilization, the six replicates of the irradiated control had a mean of 0.0% (SE = 0.0) eggs fertilized and therefore none of the eggs had embryo development. Between 19 and 24 hours post-fertilization, the six replicates of the non-irradiated control had a mean of 76.0% (SE = 4.5) of eggs fertilized and 65.3% (SE = 7.0) of those fertilized eggs had identifiable embryo development (Table 4.1).

Table 4.1. Number of fertilized/activated and non-fertilized/activated eggs per 40 eggs sampled and percent fertilized/activated eggs and embryo development by treatment between 19 and 26 hours post-fertilization.

Treatment	Time post-fertilization/ activation (hours)	# fertilized eggs/sample (± SE)	non-fertilized eggs/sample (± SE)	% fertilized eggs (± SE)	% embryo development (± SE)
Non-irradiated	19–24	24.5 (2.0)	7.7 (1.4)	76.0 (4.4)	65.3 (7.0)
Irradiated Only	21–22.5	14.5 (1.4)	16.5 (0.9)	46.6[a] (3.8) (Activated)	0.0 (0.0)
Irradiated and Pressure Shock	19–26	14.8 (5.4)	17.2 (5.1)	40.9 (15.3)	8.9 (4.2)
Irradiated and Cold Shock	19.5–26	13.6 (3.5)	16.6 (3.9)	44.8 (11.7)	8.9 (4.2)

[a]Irradiated sperm did not fertilize eggs.

Between 19.5–26 hours post-activation, the six replicates of the irradiated cold-shock treatment showed a mean of 44.8% (SE = 11.7) of eggs activated and 8.9% (SE = 4.2) of those eggs had discernable embryonic development to the notochord stage. Between 19–

26 hours post-activation, the six replicates of the irradiated pressure-shock treatment had a mean of 40.9% (SE = 15.3) of eggs activated and 8.9% (SE = 4.2) of those eggs had discernable embryonic development to the notochord stage. The non-irradiated control demonstrated the highest rate of fertilization and the highest percentage of identifiable late-stage embryos. The irradiated cold-shock treatment and irradiated pressure-shock treatments had similar egg activation rates and embryonic development rates. There was a significant (F = 36.73, df = 2, P = <0.001) difference between the mean embryo development of the non-irradiated control and the irradiated pressure-shock and irradiated and cold-shock treatments between 19 and 24 hours post-fertilization/activation (Fig. 4.2).

At 48 hours post-fertilization/activation there was no significant (F = 2.68, df = 2, P = 0.122) difference between the mean embryonic development of the non-irradiated control, irradiated pressure-shock, and irradiated cold-shock treatments. The control had a mean embryo survival of 17.3% (SE = 6.6; Table 4.2). The irradiated pressure-shock treatment had a mean embryo survival of 5.8% (SE = 2.7) and the irradiated cold-shock treatment had a mean embryo survival of 1.3% (SE = 0.9). There was no embryo development with the irradiated only sperm. The irradiated control had 0.0% (SE = 0.0) embryo development 48 hours post-fertilization/activation, thus indicating UV irradiation successfully bound the genetic material of the sperm to the plasma membrane rendering its contributions to the fertilization process incompatible. The irradiated sperm merely activates egg development and cellular division without contributing genetics materials from the male. However, there was no division without

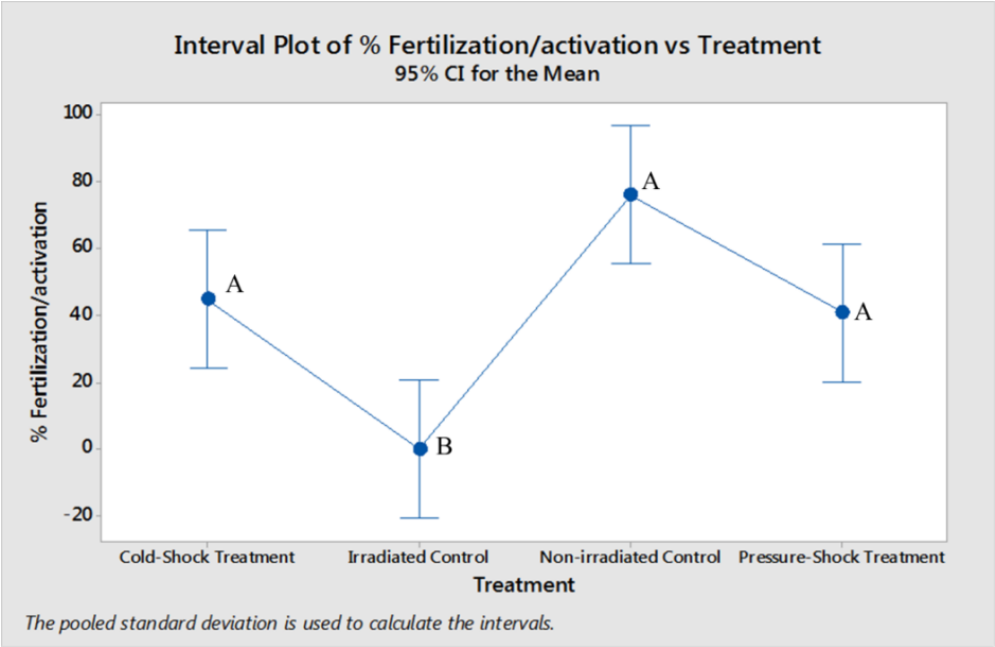


Figure 4.2. Mean percent egg fertilization/activation and 95% CIs for cold shock treatment, irradiated control, non-irradiated control and pressure-shock treatment between 19 and 24 hours post-milt addition (Points with similar letters are non-significant).

contributing genetics materials from the male. However, there was no statistical difference in the mean embryo survival between the non-irradiated control and irradiated pressure shock and irradiated and cold-shocked treatments (Fig. 4.3). Fish were grown through metamorphosis and both non-irradiated control and irradiated pressure-shock treatment had surviving larval stocked at day 58 post-hatch.

Table 4.2. Results of embryo survival (percent) by treatment, 48 hours post-fertilization/activation.

Treatment	Time post-fertilization/activation (hours)	Percent embryo survival (± SE)
Irradiated Control	48	0.0 (0.0)
Non-Irradiated Control	48	17.3 (6.6)
Irradiated Pressure Shock	48	5.8 (2.7)
Irradiated Cold Shock	48	1.3 (0.9)

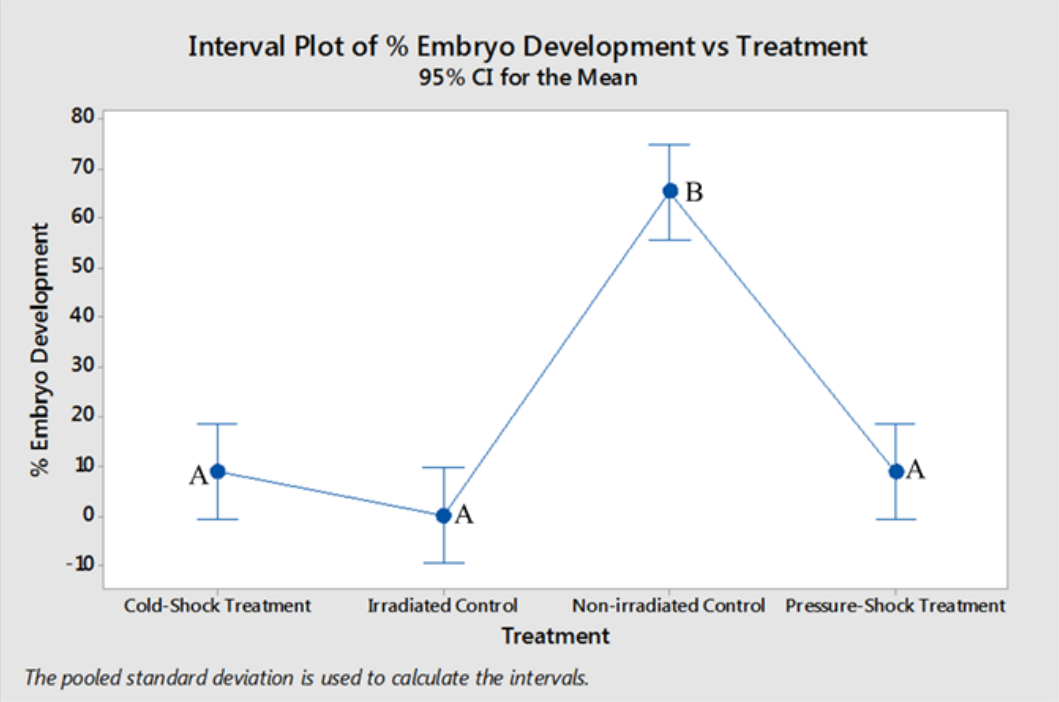


Figure 4.3. Mean percent embryo development rates and 95% CIs for control, cold shock, and pressure shock treatments between 19 and 24 hours post-fertilization/activation (Points with similar letters are non-significant).

A multivariate analysis of variance (MANOVA**)** and Wilk's criterion test of the General Linear Model performed to model the relationship between percent fertilization/activation, percent embryo production, and treatment found a significant (F = 15.19; df = 6,38; $P < 0.0001$) effect in treatments with the non-irradiated control being different from the cold-shock or pressure-shock treatment (Fig. 4.4). An adjusted R-square accounted for 84.4% of the variability.

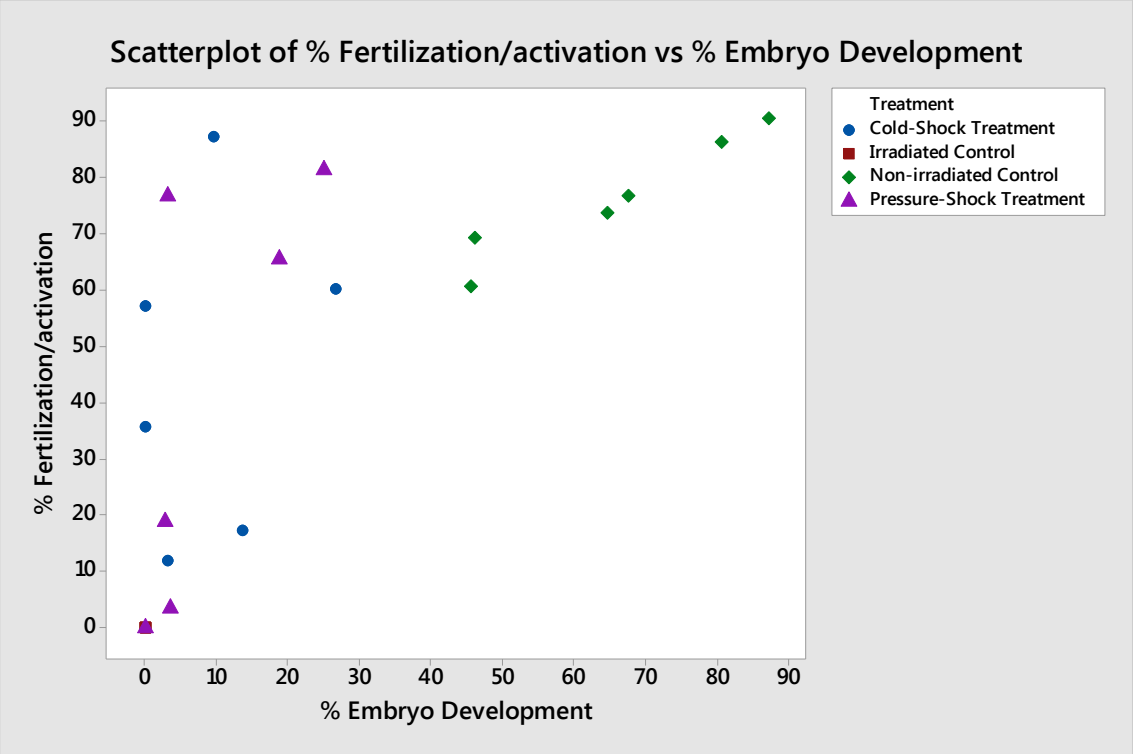


Figure 4.4. A scatterplot of percent fertilization/activation vs percent embryo for irradiated control, non-irradiated control, cold-shock treatment, and pressure-shock treatment.

Discussion

All treatments except the control demonstrated less than 50% survival. The relationship between fertilization/activation and embryo production accounted for 65.2% of the variability between treatments, with the control being different from cold- and pressure-shock treatments. Although there was no significant difference between the irradiated cold-shock and pressure-shock treatments, there was a biological difference with the irradiated pressure-shock treatment having a 5.8% survival whereas the irradiated cold-shock treatment had only a 1.3% survival. Starting with an equal number of eggs, the irradiated pressure-shock treatment would, as a result, produce more surviving larvae. Because of this, one would need only 30–40 larvae to survive through sex reversal and then be used for broodstock, therefore, the pressure shock treatment was obviously better. While the process of using pressure- and cold-shock is time consuming and involves moderate increases in production costs, it is relatively simple to produce viable gynogenic larvae with these methods. The potential for failure lies within the egg quality of the female. If a female flounder does not produce viable eggs during strip spawning, treatments will have low success rates. Three of the females used in this study did not produce viable eggs (cloudy in color) or any eggs whatsoever. These females falsely lowered success rates of the treatments in this study due to their inability to produce viable eggs to become viable offspring. If the female produces viable eggs then treatments have higher success rates. The females that readily produced viable eggs contributed the majority of eggs to the successful treatments. This could potentially be

due to natural fecundity, better overall health, or the fact that they had not spawned yet for this particular breeding season.

The irradiated cold-shock treatment had a lower percentage of activated eggs at 24 and embryonic development at 48 hours post-activation and produced a lower percentage of larvae. This method should not be considered a preferred treatment for the production of gynogenetic clones because of the lower number of viable embryos it produces. There are several physical issues with the cold-shock methodology that may lead to this result. The process of transferring the activated eggs to the cold-water bath also could have contributed to egg damage, leading to lower numbers of viable fertilized eggs. Due to the jarring of eggs when transfer, eggs had the potential to be damaged. This problem could be mitigated by reduced handling and longer periods of cold-shock treatment at cooler temperatures (Luckenbach et al. 2004). Another potential mitigation technique would be to use a greater number of activated eggs to compensate for the damaging of eggs during handling procedures. In my study, smaller batches of eggs were used because each spawn from an individual females had to be split four ways among treatments. Luckenbach et al. (2004) found cold-shock treatment used in other marine teleosts (Felip et al. 2001) was successful to an extent, with a mean fertility value of 21.3 ± 1.3 and low embryonic development was due to increased handling required for the treatment that damaged embryos.

The irradiated pressure-shock was the most biological viable technique to produce gynogenetic clones of the female. Hydrostatic pressure-shock has proven to be effective at inducing both triploidy (Benfey and Sutterlin 1984, Chourrout 1984,

Fetherman et al. 2015) and gynogenesis, which are similar processes, in many species of fish (Pandian and Koteeswaran 1999, Ihssen et al. 1990, Peruzzi et al. 1993, Peruzzi and Chatain 2000). This is due in part because there is limited handling of the activated eggs (Luckenbach et al. 2004). Greater percentages of activated eggs and embryos were recorded 24 and 48 hours post-hatch, respectively, in the present study. Success rates were low in comparison to large-scale protocols; however, these results are typical of any gynogenesis procedure. Morgan et al. (2006) found timing of pressure-shock did not significantly affect percent activation, and survival of pressure-shocked embryos ranged from 9.6 to 50.2% in southern flounder.

Further research is needed to elucidate which female southern flounder will produce viable, high quality eggs. High quality egg-producing females need to be kept and monitored over several years. Females also need to be provided with adequate nutritionally complete diets and kept in low stress environments. The key constraint of my treatments was the viability of the eggs and the condition of the female flounder to produce healthy eggs for use in the different treatments. Spawning times also can contribute to the low embryo survival. Spawns of other fishes such as cobia have been correlated with moon cycle (Benetti et al. 2007). Investigation into the factors that determine if females will produce viable eggs during strip spawning and timing of spawning peptide application and dosage are necessary to ensure success of these treatments in future studies or production.

The process of producing gynogenetic clones only needs to be accomplished once, therefore limiting strip spawning and intensive thermal manipulation to produce

female flounder (Luckenbach 2004). A producer must only create gynogens once, sexually reverse the gynogens using 17α-methytestosterone to create super XX females that are genetically female, but physically male. The producer can then breed these super XX females with normal females collected from the wild or kept on hand at the hatchery. Because no male DNA contributes to the production of offspring from super XX females and normal females, there is a 100% guarantee of all female offspring, thus limiting the costs associated with collecting and maintaining males of the species.

Based on the results of this study, I recommend the use of hydrostatic pressure-shock to produce gynogens. Further investigation is still necessary as to how these offspring will perform once sexually reversed. It also is key that female flounder used to produce eggs for these treatments must be in good physical condition, fed complete nutrient diets prior to spawning, and be maintained in a relatively stress free environment prior to spawning events.

CHAPTER V

DEVELOPMENT OF AN ECONOMICAL TECHNOLOGY TO HARVEST, SIZE-GRADE, AND ENRICH LIVE ZOOPLANKTON FROM FERTILIZED PONDS TO FEED MARINE FISH LARVAE

Introduction

In order for the United States to expand aquaculture production of marine fish species in both land-based and offshore culture systems, the industry must first overcome the major bottleneck of severely limited production and availability of juveniles. One way to potentially increase production of marine finfish juveniles is to transition from the intensive, high-cost, equipment-and labor-intensive, monoculture production of live-food organisms that is currently used to extensive, simple, low-cost, farmer-friendly technologies that take advantage of natural productivity to deliver a diversity of enriched live-foods.

The success of hatchery production of larvae, fry, and fingerlings is dependent on the availability of suitable live-foods (Lim et al. 2003). Live-foods contain essential nutrients such as proteins, lipids, carbohydrates, vitamins and minerals and are required for achieving maximum growth and survival (New 1999). Currently, commercial larval production of most marine species relies upon culture of live-marine microalgae or purchase of suspended, stabilized microalgae paste that are fed to rotifer monocultures (Das et al. 2012). Rotifer monocultures are nutritionally inadequate for larval fish nutrition. The rotifer cultures are typically fed only one or two species of microalgae that

may not contain all of the nutrients required by fish larvae. Therefore, an additional enrichment step is required to ensure they contain proper nutrition (Cutts 2001).

After a period of days to weeks, rotifers are phased out in favor of a monoculture of *Artemia* nauplii which are larger than rotifers. Again, *Artemia* nauplii require an additional enrichment step to ensure proper nutrition is delivered to the larvae (Kolkovski et al. 2004). While rotifers can typically be generated and cultured on demand, the only source of *Artemia* nauplii is from wild capture of resting cyst. Wild populations of *Artemia* capable of supporting wild harvest are declining globally, and harvest has become much more erratic with fluctuating populations (Roberts and Conover 2013, 2014). Prices of *Artemia* cysts have increased significantly over the last 20 years as demand has increased, and most of the wild sources cannot support the growing demand for culturing fish larvae and other competing uses (Zarela 2010). At some point during or after the transition to *Artemia* nauplii, commercial larval diets are introduced to transition larvae onto less labor-intensive, more nutritionally complete diets.

The complicated and expensive process of providing live-food organisms for larviculture often makes juvenile production economically non-viable for most producers, or simply precludes them from attempting live-foods production due to complexity of the process and labor intensity (Das et al. 2012). It is arguable these intense culture methods have not resulted in any major marine food-fish production industries in the United States, although small, but non-sustained production and marketing of several marine food-fish species such as cobia (*Rachycentron canadum*),

southern flounder (*Paralichthys lethostigma*), and Florida pompano (*Trachinotus carolinus*) have occurred using these methods. The economic cost of intensive systems for larval production is a key bottleneck preventing widespread production of marine fish larvae.

Pelleted microdiets have long been touted as the remedy to this restriction associated with use of live-foods yet they often lead to growth retardation and skeletal abnormalities (Person Le Ruyet et al. 1993). There are many marine species of potential commercial importance, such as the red (*Lutjanus campechanus*) and mangrove (*Lutjanus griseus*) snappers, and spotted seatrout (*Cynoscion nebulosus*), in which early or quick transition to artificial diets has so far proven to be non-feasible. The prevailing theories in the scientific community are these species may require the natural movements (angular velocity) provided by live-food organisms (Mussi et al. 2005, Jackson and Lenz 2016, Bruno et al. 2018), or the presence or excretion of specific chemicals to trigger the strike and feeding instincts of these larvae (Valentinčič et al. 1999). In fact, the movement of live-food organisms, such as *Artemia* nauplii, has been demonstrated to increase the consumption of simultaneously offered microdiet pellets by up to 120% in some marine fish such as gilthead seabream (*Sparus aurata*), indicating the strong need for visual and movement cues to stimulate feeding in larval fish (Kolkovski et al. 1997). Total replacement of live-prey for larval marine fish is reported to be impossible (Person Le Ruyet et al. 1993).

Several marine fish species of commercial importance, such as red drum (*Sciaenops ocellatus*), spotted seatrout, and cobia are able to be cultured using extensive

production techniques (Colura et al. 1976, Lyon and Fisher 1998, Weirich et al. 2004, Schwarz 2004, Holt et al. 2007, Cason and Anderson 2015). Fertilized eggs, or more typically newly hatched larvae, are stocked into newly filled and fertilized ponds (Cason and Anderson 2015). The larvae receive suitable nutrition by preying upon a diversity of zooplankton from natural production and greater numbers of fish are able to be produced than with live-foods production (Cason and Anderson 2015, Sink et al. 2018). The zooplankton of natural production includes a mixture of multiple species of protozoa, copepods and other arthropods, rotifers, cladocerans, insects eggs and larvae, etc. This is a practical, low-cost method of production for red drum (Cason and Anderson 2015). This extensive method has resulted in red drum becoming the only true marine finfish species to achieve sustained commercial culture in the United States (USDA 2013). Anadromous striped bass (*Morone saxatilis*) and hybrid striped bass (*M. Saxatilis* X *M.* spp) are often cultured using similar extensive pond-production methods, although a brief (<7 days) live-feeding period in a hatchery is sometimes used prior to pond stocking of larvae (Geiger et al. 1985).

Extensive pond production that can be used for some marine species still has drawbacks (Davis 1990, Sink et al. 2018). Succession of the natural phytoplankton and zooplankton communities must be setback by either draining and refilling or chemical methods (typically copper-based algaecides or potassium permanganate; Geiger 1983). This starts succession over with smaller species of zooplankton of correct size for marine fish larvae, eliminates the majority of larger predacious zooplankton, reduces the number of predacious insects, and reduces the amount of potentially harmful plankton that

produces toxins such as certain cyanobacteria species (Davis 1990). Ponds must then be fertilized using inorganic fertilizers at the appropriate time prior to stocking larvae, approximately 10 days prior for red drum, so that a "bloom" of the correct-size zooplankton for larval feeding is present when the fish are stocked (Davis 1990, Sink et al. 2018).

If pond filling or fertilization is too late, insufficient live-food organisms will be present to support the larvae, and production will be poor. If the pond is filled or fertilized too early, numerous zooplankton too large for the larvae to eat will result, and large numbers of predacious zooplankton and insects can develop in the pond (Davis 1990, Sink et al. 2018). In addition, small changes in temperature or available sunlight can dramatically alter zooplankton production and succession, as well as subsequent larval survival. For many marine species, up to 2.4 million larvae may be stocked per hectare to return yields of only hundreds of juveniles per hectare due to environmental fluctuations, such temperature and/or salinity, or from predation by zooplankton, insects, birds, fish, and other predators (Anderson et al. 2016). Bird predation can be extremely high for many marine fish larvae including red drum (Sandifer et al. 1993).

There is potential to develop a new type of simple technology for hatcheries that eliminates the high-costs, and extensive technology and labor requirements typically required for intensive marine larviculture. This new technology also would eliminate unnecessary water waste from draining larval ponds, uncertainty in timing of fertilization, and high mortality due to predators and environmental fluctuations during

extensive larval culture. This new system would constitute a type of hybrid technology, between intensive and extensive systems.

The system would use factors from intensive-culture systems that would keep larval fish concentrated in tanks where they are easily visible, safe from predators, easy to manage, and with greater environmental control, while taking advantage of the massive live-foods production from natural productivity and diversity of prey items of extensive pond systems. Numerous facilities along the Gulf and Atlantic coasts with available saline waters are poised to take advantage of this simple to operate, automated, live-foods harvesting system with the ability to size-grade live-foods for each specific growth phase of a fish species, if the potential technology is developed. This technology is not limited to any single species and can be used to culture any species from baitfish such as Atlantic croaker (*Micropogonias undulates*) to foodfish such as tripletail (*Lobotes surinamensis*) with little modification.

Sapkota et al. (2012) developed an outdoor mobile fish nursery (MFN) system to culture freshwater larvae until large enough to be transferred to fertilized ponds. The trailer-mounted MFN's purpose was to provide larval fish with live-foods harvested from production ponds by moving the system, fish tanks, and larvae to the pond with the best zooplankton production at the time. The MFN did this using paired drum filters with a 180-µm screen to remove zooplankton larger than 180 µm, and a 60-µm screen to retain zooplankton between 60 and 180 µm while removing zooplankton smaller than 60 µm. The appropriately sized zooplankton (<60–180 µm) were then washed into a

holding chamber and fed continuously to larval tanks using a low-volume dosing-pump, thus ensuring larvae received a constant supply of appropriately sized live-foods.

Preliminary trials of the TFN at the University of Arkansas at Pine Bluff using goldfish (*Carassius auratus*), hybrid striped bass, and hybrid crappie (*Pomoxis* spp.) all proved the system could successfully produce food from fertilized ponds to ensure adequate nutrition. This system reduced costs regularly associated with the live-food production typically needed to ensure larval grow out (Sapkota et al. 2012). Though this system has proved effective for freshwater fish species, no system has been developed for larval production of marine fish species.

Objectives and Hypotheses

This project will develop a new, indoor hatchery-based technology, partly based on the TMN. The new technology will then be tested to investigate the feasibility of automated live-food-harvest (LFH) systems in marine larviculture. I intend to investigate the utilization of natural live-food production from fertilized, phytoplankton- and zooplankton-seeded, marine ponds. This LFH system should reduce costs, equipment, time and labor requirements of intensive culture systems, while eliminating the uncertain timing of fertilization and high mortality due to predation and environmental fluctuations of extensive culture of marine finfish larvae. The hypothesis was that a new technology could be developed to harvest live-food organisms of the correct type and size from outdoor production ponds in sufficient quantities to feed difficult to rear marine finfish larvae, thus reducing dependence on live-food monoculture production.

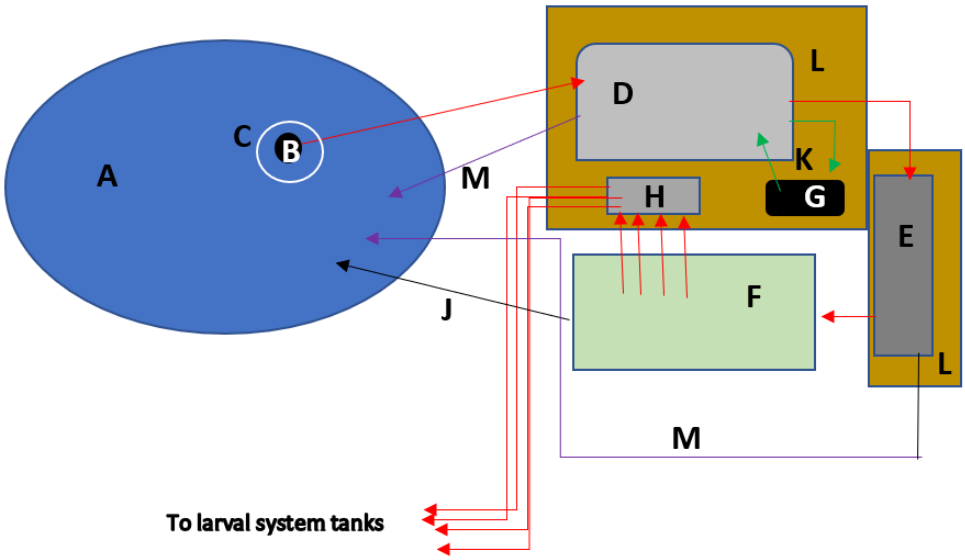
Methods and Procedures

To determine if a LFH system was as efficient at growing spotted seatrout larvae as the current method of indoor hand feeding zooplankton and prepared diet, I compared continuous flow-through with fertilized and seeded pond water (C, control) with a continuous LFH system (LFH, treatment 1) and a traditional enriched-rotifer monoculture (ERM, treatment 2). Each control (C, $n = 4$) and treatment (LFH, $n = 4$; ERM, $n = 4$) was replicated four times in the form of 12 larval production tanks. Spotted seatrout were chosen as the test species because it is a common and popular marine species for angling and as a foodfish, is a candidate species for commercial aquaculture and marine stock enhancement programs (Garlock et al. 2017), has developed hatchery methods (Wisner et al. 1996), and early or quick transition to artificial diets has so far proven to be non-feasible for this species.

Development of the Live-Foods Harvest System

A LFH system was designed and constructed for the study. The primary LFH system components consisted of the following items and the preceding letters correspond to the component located on the diagram below (Fig. 5.1, A–M). The fertilized and seeded pond (Fig. 5.1, A; total cost for succession setback and phytoplankton and zooplankton seeding USD$292.52). A continuous running 0.75-HP stainless steel submersible sump pump (Fig. 5.1, B; Model 92731, 18,170 L/hour; Superior Pump, Minneapolis, Minnesota; USD$174.59) suspended by a 61-cm G-series life ring buoy (Fig. 5.1, C; Jim-Buoy, North Hollywood, California; USD$44.99) 30 cm below the pond surface to pull water from the pond containing zooplankton. A solar light was

placed to shine directly on the life ring buoy to attract zooplankton overnight. A drum filter (Fig. 5.1, D; model ProfiDrum Eco 45/40, ProfiDrum USA, Scandia, Minnesota; 25,000 L/hour capacity at 70 um filter size; USD$4,100.00) to screen unwanted larger



A. Outdoor, seeded, marine pond
B. Sump pump
C. Circular buoy to suspend pump at surface
D. Drum filter
E. Parabolic filter
F. Live-foods collection & enrichment tank
G. Drum filter cleaning pump
H. Programmable auto dosing pump
I. Red lines indicate flow of water in hoses through LFH system
J. Black lines indicate filtered water flow through hoses back to pond
K. Green lines indicate water flow from drum filter to automated cleaning pump
L. Mobile tables on casters
M. Purple color lines indicate flow of over-sized zooplankton and pond debris to external drain

e;

Figure 5.1. Component and operation diagram of a live-food harvest system developed to culture marine finfish larvae during the study.

sizes of zooplankton and remove them from the pond water. Which is connected to a parabolic filter (Fig. 5.1, E; Model 2875, 265 L/minute; FIAP, Pentair Aquatic Eco-Systems, Inc., Apopka, Florida; USD$2,068.50) to concentrate rotifers, copepods and other live-foods and then flows to a 189-L plastic water storage tank (Fig. 5.1, F; Model CRMI-50VTFWG, The Tank Depot, Houston, Texas; $78.99) to collect live-foods of the desired size where nutrient-enrichment solutions could be applied. An automated internal self-cleaning pump to clean the drum filter (included with the ProfiDrum Eco 45/40). Fig. 5.1 G) A DP-4 programmable auto dosing pump (Fig. 5.1, H; Jebao®, Guangdong, China; $69.99**)** to deliver the enriched live-foods to larval tanks. All major components were mounted on two small, wooden, movable tables mounted on casters (Fig. 5.1, L; 91 cm by 107 cm and 46 cm by 76 cm; USD$142.17). Filtered water from the rotary drum and parabolic filter was then routed to a marsh area to aid in pond succession of the appropriate size zooplankton (Fig. 5.1, M). Various generic garden hoses, tubing, and PVC fittings were installed to route water from the pond around the system, tanks, and drains (Fig. 5.1; I, J, and K; $148.61). Total system costs including succession setback and phytoplankton and zooplankton seeding were USD $7,120.36. Component parts of the live-foods harvest system are pictured in Figure 5.2.

Pond Succession Setback and Seeding of Live-Marine Organisms

This research used an EPDM rubber-lined (Firestone 45 mil EPDM Rubber Pond Liner, Bridgestone Americas, Nashville, Tennessee) 0.05-ha pond normally used to culture marine larvae using extensive pond culture methods. For this study, instead of stocking the pond with larvae, it was fertilized, seeded with live-phytoplankton and

zooplankton, and used as a live- foods culture pond. Pond succession was set back by applying a chelated copper algaecide (Cutrine®Plus, Applied Biochemists, Germantown, Wisconsin) according to the label to kill the planktonic algae present. Two days later, the pond was fertilized using inorganic fertilizers (12-48-8; Perfect Pond Plus® Mossy Oak Bio Logic, West Point, Mississippi), 19 days prior to larvae stocking.

Figure 5.2. Photograph of component parts used in the live-foods-harvest system. Letters correlate to Figure 5.1 diagram.

After fertilization, the pond was immediately seeded with 2 L of live, concentrated *Nannochloropsis* spp. (Mercer of Montana, LLC, Billings, Montana) and 1

L of live, concentrated *Tetraselmis* spp (Mercer of Montana, LLC, Billings, Montana) marine algae. Five days later, the ponds were seeded with 20 million *Brachionus plicatilis* rotifers and 5 million *B. rotundiformis* rotifers (Reed Mariculture Incorporated, Campbell, California) and 25,000 *Parvocalanus crassirostris* (Reed Mariculture Incorporated, Campbell, California). Pond seeding was done to ensure the pond plankton community was dominated by phytoplankton containing the vital nutrition required by marine fish larvae and zooplankton of the correct sizes to deliver the nutrition to the fish larvae.

Egg Acquisition

Fertilized spotted seatrout eggs were collected from the Sea Center Texas hatchery in Lake Jackson, Texas and transported via oxygenated bags to the Aquaculture Research and Teaching Facility (ARTF; 13950 FM 60 East, Somerville, Texas 77879). Sea Center participates in a spotted seatrout stock enhancement program and supplied 3,000 spotted seatrout eggs for this research. Eggs were collected from their recirculating hatch tanks and measured using a graduated cylinder with an estimated egg volume of 1,800 eggs per mL. Eggs were then placed in a plastic bag filled with 28 ppt seawater and filled the rest of the way with compressed oxygen. Eggs were then double bagged and placed in an insulated cooler for transport to the ARTF. Approximately 10% of the eggs had hatched by arrival at the ARTF.

Stocking and Larvae Production Tanks

Eggs and newly hatched larvae were equally divided among 12, 113-L recirculating larval production tanks to be used for the study at a density of 2.2 eggs/L (250 eggs per tank). Mean hatch rate was approximated at 63% and mean larval survival to first feeding at 2 days-post-hatch was approximated at 71%, yielding a mean of 118 larvae per tank to start the trial. Two treatments and a control were each randomly assigned to four replicate tanks of larvae. The incubation room was set for a 12-hour light and 12-hour dark schedule. The incubation room temperature was maintained at 23°C. Light aeration was maintained in each tank for the duration of the experiment. Water exchange was increased from 50% per day to 100% per day at 3 days-post-hatch. Tanks were siphoned every other day starting at 4 days-post-hatch until 30 days-post-hatch.

Control Treatment

Four tanks in the larval room were assigned to the control treatment and set up as flow-through systems. The control tanks received non-filtered pond water supplied from the fertilized and seeded pond at a rate of 0.05 L per minute and was gradually increased to 1 L per minute over the course of 1 week. The control treatment simulated extensive fertilized pond production, but was limited in this capacity as it eliminated most insect, bird, and fish predation and maintained fairly stable environmental temperatures as the culture room temperature was controlled at 23°C. However, water temperature still varied more for this treatment due to daily and diurnal pond water temperature changes and higher pond water exchange than for other treatments.

Water was pumped directly from the pump in the salted pond with a 15-m garden hose. This hose fed into a PVC pipe manifold with water outlets to each control tank. Each tank contained a slotted internal standpipe covered with 250-µm nylon mesh and an external standpipe that maintained constant water volume. Water flowed through the standpipes and was returned to the seeded pond via a PVC drainpipe. Spotted seatrout larvae were allowed to consume any phytoplankton or zooplankton that was pumped through the tanks from the pond and continuous water flow constantly renewed the availability of plankton. Water in the tanks was observed to be green to green-brown in color from suspended plankton and contained filamentous algae growth. The water supply valves to each tank were cleaned daily using a pipe brush to ensure uninterrupted water flow and plankton supply.

Live-Foods-Harvesting-System Treatment

The LFH system treatment consisted of four larval tanks operated as a recirculating aquaculture system. The system included a 568 L fiberglass sump, 1 horsepower centrifugal pump (Harris H1572729 ProForce 1 HP, Harris Pool Products, Kenosha Wisconsin), sand filter (Ariasd 4000© Pentair Aquatic Eco-Systems Inc. 2395 Apopka Blvd. Apopka, Florida), canister filter (Water Co.©, Augusta, GA), and UV filter (a Jebao© PU-36 UV clarifier Jebao®, Guangdong, China). Each tank contained a slotted internal standpipe covered with 250-µm nylon mesh and an external standpipe that maintained constant water volume. Wastewater flow from the tanks was returned to the sump for filtration. Tank temperature was maintained by room temperature at 23°C, salinity was maintained at 28 ppt, and dissolved oxygen was maintained above 5 mg/L.

Larvae in the LFH system treatment received feedings of 4,000 mL of concentrated, enriched zooplankton from the live-foods collection and enrichment tank 24 times daily (hourly). The drum filter mesh of the LFH system was started at 250 µm to remove debris and zooplankton too large for the larvae to eat and the mesh of the parabolic filter that retained the correctly-sized zooplankton was started at 60 µm. At day 11 post-hatch, the mesh size of the LFH system drum filter was changed to a 400-µm mesh to allow larger zooplankton to be fed to larval fish, and the mesh of the parabolic filter stayed constant at 60 µm to support any slow growing larvae. Once daily, 10 mL of N-Rich PL Plus® enrichment solution (Reed Mariculture Inc., Campbell, California) was added to the live-foods collection and enrichment tank. Zooplankton in the holding tank of the LFH system were enumerated and recorded daily using a 1-mL sample, microscope, and graduated counting slide. In addition, a 1-mL sample of larvae tank water was enumerated and recorded daily using the same methods. The LFH system provided approximately 40,000 rotifers, copepods, and other food organisms to each tank at each feeding (24 X daily). A 50 µm zooplankton filter was used to assess pond production and enumerate rotifers and copepods from a filtered, 1-mL sample of pond water and recorded.

Enriched-Rotifer-Monoculture Treatment

The enriched-rotifer-monoculture (ERM) treatment included four tanks of larvae in a recirculating system that were fed an intensive monoculture of *B. plicatilis*, followed later by a monoculture of *Artemia* nauplii and a commercial larval diet (Otohime Marine Weaning Diets, Biokyowa, Japan). The enriched-rotifer monoculture treatment system

included a 568-L fiberglass sump, 1-horsepower centrifugal pump (Harris H1572729 ProForce 1 HP, Harris Pool Products, Kenosha, Wisconsin), sand filter (Ariasd 4000© Pentair Aquatic Eco-Systems Inc. 2395 Apopka Blvd. Apopka, Florida), cannister filter (Water Co.©, Augusta, Georgia), and UV filter (a Jebao© PU-36 UV clarifier Jebao®, Guangdong, China). Each tank contained a slotted internal standpipe covered with 250-µm nylon mesh and an external standpipe that maintained constant water volume. Wastewater flow from the tanks was returned to the sump for filtration. Tank temperature was maintained by room temperature at 23°C, salinity was maintained at 28 ppt, and dissolved oxygen was maintained above 5 mg/L.

Starting at day 2 post-hatch, larval fish in the enriched-rotifer monoculture treatment tanks were fed cultured, enriched rotifers at a rate of 100 rotifers per mL, twice daily, ending day 19 post-hatch (Daniels and Hodson, 1999). Prior to rotifer feeding, rotifers were enriched using 10 mL of N-Rich PL Plus® (Reed Mariculture Inc., Campbell, California).

Starting on day 6 post-hatch, larval fish in all control and treatment tanks were supplementally fed 1 g of a commercial dry larval diet (Otohime diet B1, Biokyowa, Japan) three times daily (Daniels and Hodson, 1999). Starting day 13 post-hatch, larval fish were fed 1,000 *Artemia* nauplii per tank twice daily (Daniels and Hodson, 1999). Artemia feeding ended on day 30 post-hatch (Daniels and Hodson, 1999). Metamorphosed juveniles from each tank were completely harvested and enumerated and measured to determine survival and growth at 4 weeks-post-hatch.

Data Analysis

The SPSS® software platform was used for all data analyses. Data for each control and treatment were analyzed using a one-way analysis of variance (ANOVA). A $P \leq 0.05$ was used to indicate statistical significance among treatment means. When significant differences among means were found, treatment means were separated using Duncan's multiple-range post-hoc test. The alpha for the power analysis was set at 0.05 and the power of the test was set at 0.90 to generate sample size. In addition, a General Linear Model was performed to model the relationship between number of fish surviving and size (cm) of these fish by treatments.

Results

The LFH system provided rotifers, copepods, and infrequently other food organisms at each feeding (Table 5.1). This volume of live-food organisms was available to approximately 100 larvae in each tank. In each of the Control tanks, an average of 11 (SE = 1.47) rotifers per mL at 6 L/hour for 24 hours a day provided an average of 15,840,000 rotifers daily. In each of the LFH tanks, an average of 8 (SE = 0.66) rotifers per mL at 4,000 mL/hour on the hour provided an average of 768,000 rotifers daily. In each of the enriched-rotifer-monoculture tanks, an average of 100 (SE = 10.31) rotifers per mL at 2,000 mL (twice a day) provided an average of 400,000 rotifers daily. In addition, an average of 1.5 (SE = 0.29) copepods per mL at 6 L/hour for 24 hours a day provided an average of 216,000 copepods daily per control tank. And an average of 0.3 (SE = 0.12) copepods per mL at 4,000 mL/hour on the hour provided and average of 28,800 copepods daily per LFH tank.

Table 5.1. The number (*n*) of samples taken, mean number and standard error of rotifers and copepods observed per mL of water supplied to each of the 4 tanks within each treatment.

	Mean number of rotifers			Mean number of copepods	
Treatment	*n*	$\bar{x}$	SE	*n*	$\bar{x}$ SE
Control	4	11.0	1.47	4	1.5 0.29
Live-Foods-Harvesting-System	30	8.0	0.66	30	0.3 0.12
Enriched-Rotifer-Monoculture	30	100.0	10.31	None in supplied food	

The mean number of fish surviving (Table 5.2) in the four control tanks ($\bar{x}$ = 2.75, *n* = 11, SE = 1.38), the four ERM tanks ($\bar{x}$ = 3.00, *n* = 12, SE = 1.08), and four LFH tanks ($\bar{x}$ = 12.50, *n* = 50, SE = 4.77) was not significantly (*F* = 3.59, *df* = 2, *P* = 0.071) different (Fig. 5.3). However, the mean size (cm) of fish (Table 5.1) of the control larvae ($\bar{x}$ = 1.964 cm, *n* = 11, SD = 0.520) were significantly (*F* = 5.99, *df* = 2, *P* = 0.004) larger than larvae for ERM treatment ($\bar{x}$ = 1.432 cm, *n* = 50, SD = 0.4753), while larvae in the LFH treatment ($\bar{x}$ = 1.550 cm, *n* = 12, SD = 0.323) were intermediate

in size and not significantly different from any other group (Fig. 5.4). A MANOVA and Wilk's criterion test of the General Linear Model performed to model the relationship between number of fish surviving and size of these fish indicated no significant (F = 1.497; df = 4,16; $P < 0.250$) relationships in treatments (Fig. 5.5).

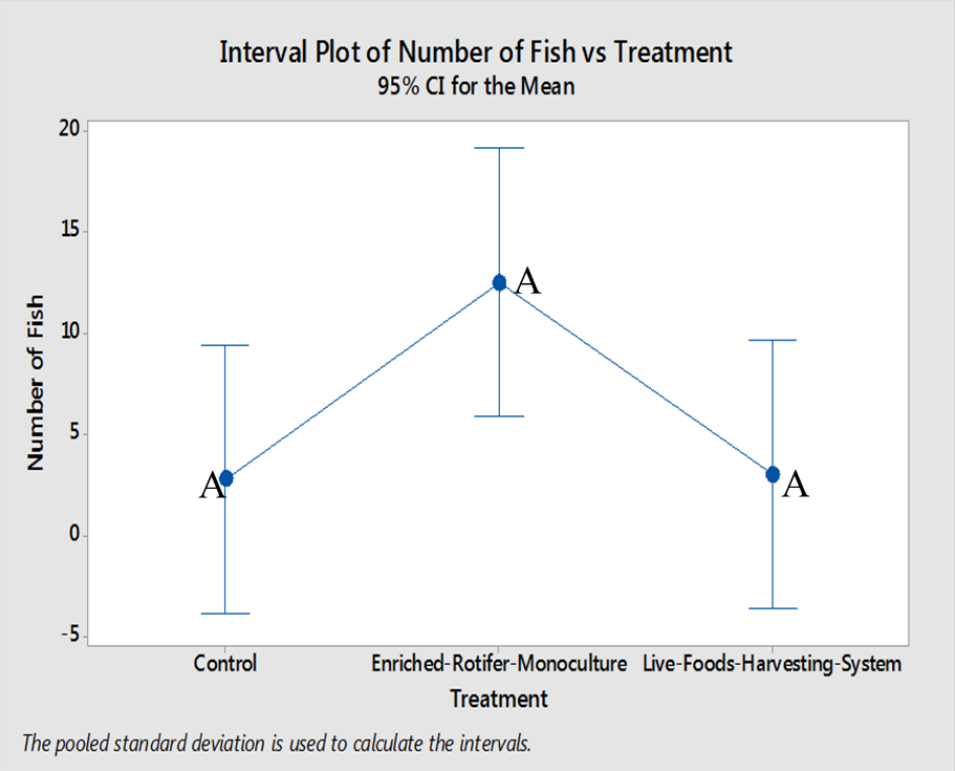


Figure 5.3. An interval plot of mean number of fish surviving by treatment (Points with similar letters are non-significant).

Table 5.2. The total, mean, and standard error of the mean (SE) of the number of fish produced and total, mean, and standard error of the mean (SE) of the size (cm) of fish by treatment.

	Number of fish produced			Mean size of fish		
Treatment	*n*	$\bar{x}$	SE	*n*	$\bar{x}$	SE
Control	11	2.75	1.38	11	1.964	0.520
Live-Foods-Harvesting-System	12	3.00	1.08	12	1.550	0.323
Enriched-Rotifer-Monoculture	50	12.50	4.77	50	1.432	0.475

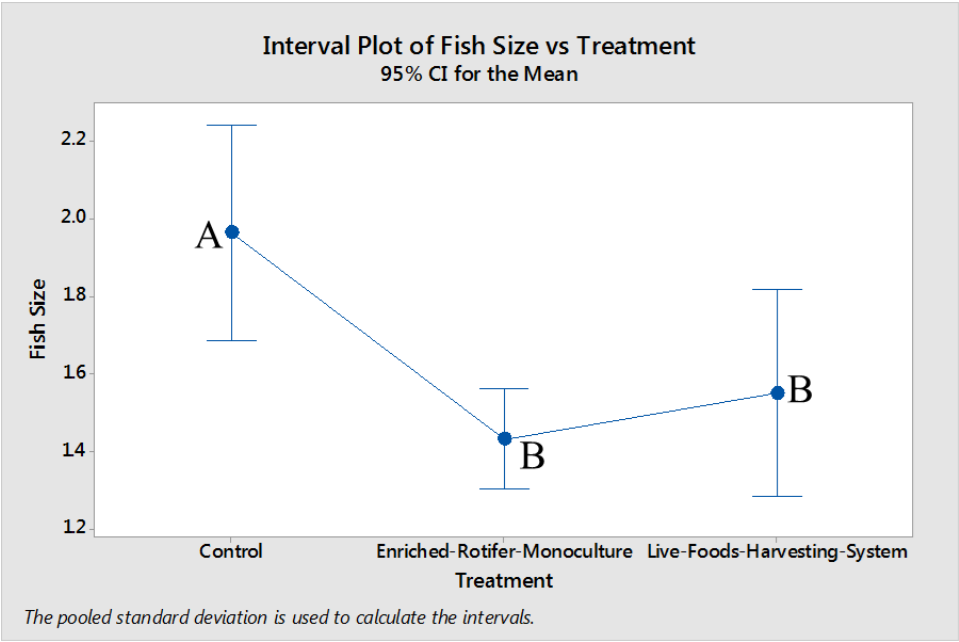


Figure 5.4. An interval plot of mean size (cm) of surviving fish by treatment (Points with similar letters are non-significant).

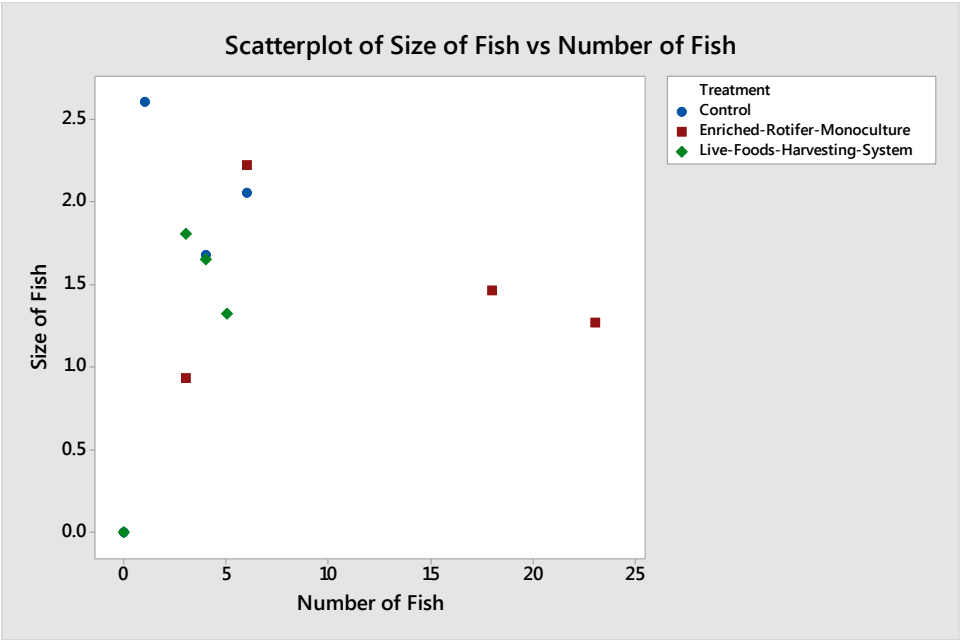


Figure 5.5. The relationship between the mean number of surviving fish and the mean size (cm) of the surviving fish.

Discussion

While the LFH system provided a feasible alternative to the individual cultivation of live-foods (e.g., algae, rotifers, and *Artemia*) the resulting data did not demonstrate significant differences in number of fish produced from each treatment. It is important to note that only one other LFH system has been tested and the results of that study were more focused on the functional success of the LFH system itself to produce a reliable amount of zooplankton rather than equipment costs (Sapkota et al. 2012).

The prior LFH system/mobile nursery constructed by Sapkota et al (2012) had an overall cost of $35,190, while my LFH cost was $7,120.36. Barring mechanical issues

discussed further in this section, the lower economic cost of my LFH system is entirely more feasible for producers to use during larval cultivation.

It should be noted that water from the seeded pond was used in both the LFH and the control treatment. The pond, when sampled, remained dominated by nannochloropsis algae and *Branchionus* rotifers throughout the study. The selection and removal of the larger zooplankton by the LFH likely altered the succession of the pond and kept the production of the highly desirable live-food organisms to a desirable size through the return of waste water from the system.

The mean number of rotifers provided in the enriched-rotifer-monoculture system was higher than provided to the LFH system and to the control treatment. The control treatment was only sampled weekly for rotifers and samples were taken directly from pond water. The continuous flow of this water containing rotifers from the ponds directly into the tanks, gave larvae an unlimited supply of food 24 hours a day. At a rate of 6 L per minute, which was the calculated water flow into the tank, 24 hours a day that is >15 million rotifers provided to each tank per day. The mean number of rotifers provided by the LFH system was 8 rotifers per mL and the system provided 4,000 mL 24 times a day. Approximately 768,000 rotifers were provided to each tank every 24-hour period. The enriched-rotifer monoculture provided an average of 100 rotifers per mL and each feeding was 3,000 mL per day, equating to 600,000 rotifers fed twice a day to each tank. The difference in rotifer and copepods (the enriched-rotifer monoculture tanks received no copepods) numbers provided the control and different treatments may have contributed to the larvae size and number discrepancy among treatments. In other

studies, common feeding amounts were between 8–10 rotifers per mL and the amount was based on the number of larvae per tank (Tucker 1996, Manley et al. 2014).

The resulting data indicated the size (cm) of the fish remaining after the 4-week trial was significantly different between the control and LFH system and the intensive monoculture production system. The LFH an system and the intensive monoculture production system demonstrated a very similar average size of larvae after the 4-week trial ended. There are several possible reasons for these outcomes.

First, the control fish were provided continuous food and not hindered with complications that arose from the LFH system. The control treatment specimens were significantly larger than the LFH and ERM treatments. These fish were allowed to feed until satiation with no limitations on the amount of food entering the system. These results correlate with the findings of Naas et al. (1992) in a green water (comparable to control treatment) versus clear water trial using larval halibut (*Hippoglossus hippoglossus*). Naas et al. (1992) determined that both growth and survival were higher in green water tank systems and that green water induced more successful search, capture, and potential utilization of prey organisms. This could be caused by the ready availability of algae for larger zooplankton to feed upon and the sustainable microculture of zooplankton that this phenomenon creates in each tank. Other studies have demonstrated the positive correlation of green water and growth and survival of marine fish larvae (Houde 1975 1978, Moffatt 1981, Chesney 1989).

The control treatment at times did necessitate extra attention and cleaning. At times the hose used in the control system that fed into the waterspouts for each tank

often became clogged with algae and detritus which limited water flow and probably continuous food for the fish. The hose had to be cleaned multiple times a day. The standpipes in each tank also often became clogged with algae and detritus and had to be switched out and scrubbed multiple times a day and oftentimes when left unattended (overnight) would overflow the tanks leading to mortality of larval fish resulting in less survival for this system.

Second, LFH system required daily maintenance and faced fouling and mechanical complications that inhibited the ability of the system to produce large enough numbers of zooplankton to maintain healthy populations of fish larvae. Due to the novel nature of this system there is very little literature to support the detrimental effects of fouling in a live-foods-production system. A comparable association could be inferred from fouling encountered in in-pond raceways, in pond cages, or marine net pens and cages. Algae that commonly contribute to fouling in the aforementioned situations are referred to as epiphytes (Smith et al. 1984, Rueness and Fredrickson 1989, Friedlander et al. 1990, Cancino et al. 1987). Epiphytes include members of the three main microalgal groups (Chlorophyta, Phaeophycae, Rhodophyta), diatoms, and blue-green algae (Fletcher 1995).

The sump pump located in the pond often got clogged with filamentous algae and needed to be cleaned daily. The hose that fed the pond water from the sump pump to the Profidrum filter often experienced clogs and algae buildup, even with daily sump pump cleanings. The Profidrum filter had a self-cleaning mechanism that required manual start up multiple times a day and the screen often was clogged with detritus, limiting the

amount of water that progressed to the parabolic filter. The parabolic filter-screen-mesh size was probably too large and without some buildup of detritus, it would allow the rotifers and water to flow back into the pond. If the screen was fouled beyond the point where the water flow was constant, there was an overflow of detritus-filled water that made its way to the holding area. The screen of the parabolic filter required cleaning daily, and even then, directly after cleaning it would only allow a small number of rotifers to make their way to the holding tank. The final issue was the peristaltic pumps that fed the filtered zooplankton to the tanks. These pumps ran for almost 24 hours straight and had to be replaced every week due to malfunction of the pumps. The tubing lines that fed the zooplankton from the holding tank into the pump and directly to the larval incubation tanks also had issues such as crimping and clogging from filamentous algae. It was observed in one tank that water flow from the holding tank to the larval incubation tank was so low that no rotifers were viable in multiple samples of the tank water. It should be noted that the 4,000 mL of water fed into the LFH system tanks did not contribute significantly to water clarity, but that tanks fed by the LFH system had much less detritus and algal build up compared to the control tanks. The water fed from the LFH system contributed to plankton build up in the tanks when compared to the observed extreme water clarity in the intensive monoculture tanks.

I believe the complications that arose from biofouling and malfunction could easily be refined to make the LFH system work. Further investigation into water filtration before it enters the system is necessary. It also would be beneficial to test the system using water from a seeded-fertilized tank, where issues would be easier to

control, rather than a pond that was exposed to the elements and left uncontrolled in regard to fouling organisms.

Lastly, the ERM did not face the biofouling issues of the control or LFH. This led to water clarity that may have exacerbated cannibalism in the tanks, although cannibalism among larval spotted sea trout is well documented and could explain the low numbers of fish at the end of the 4-week trial period (Arnold et al. 1978, Tucker 1988, Baras and Jobling 2002, Hecht and Pienaar 1993, Manley et al. 2014). In the control tanks it was impossible to visually inspect the growth of the larvae and this led to larger specimens that actively consumed tank cohorts. To remedy this issue, the trial could have been conducted for a shorter duration as cannibalism has been proven to reach an apex among larval spotted sea trout at 10 days post hatch (Arnold et al. 1978, Tucker 1988). Alternatively, the frequency of feeding and its effects on cannibalism has been researched by Manley et al. (2014). That study demonstrated that a 2-hour feeding frequency elicited the fewest aggressive and cannibalistic acts (Manley at al. 2014). Our study negates these findings as fish in the control and the LFH system tanks were fed continuously and cannibalism was prevalent.

A general-linear model was run to establish the relationship between surviving number of fish and size, but with so few fish surviving no relationship could be determined. A larger sample size would be needed to determine the relationship between cannibalism, surviving number of fish, and fish size.

CHAPTER VI

CONCLUSIONS

In conclusion, all four studies contributed to expanding technologies and methodologies during spawning and larviculture phases of marine fish hatchery production. These technologies and methodologies have application to many fish species.

The induction of ovulation through hormone injection in sexually mature adult cobia was successful. However, other studies indicate that environmental conditioning as well as hormone injections may prove to be more successful. Due to complications during this study (i.e., the presence of lesions created by numerous hormone implant injections required to achieve the desired dosage) further research was undertaken to test a novel excipient for hormone injection. The lack of fertilized eggs produced in this trial also led to the use of different species for evaluation of a live-food-harvesting system to replace conventional methodologies in live foods culture.

In the testing of a novel excipient on red drum, the excipient proved successful at limiting the adverse effects of conventional hormone implants such as Ovaplant® and Ovaprim®. Only 5% of the fish injected showed any abnormalities caused by the excipient. While this was the first clinical trial using this sucrose-based excipient, it has the potential to replace commonly used hormone implants or injections. It was minimally invasive and due to its time-release properties, it limits handling time that causes undue stress to fish. The positive implications of the results of this clinical trial

indicated this excipient can be used as a spawning aid for most species of fish with no or very few adverse effects.

Evaluating pressure- and cold-shock as viable treatments to produce gynogenetic clones in southern flounder was a small, but extremely important step in the process of producing all female southern flounder broodstock. Inducing ovulation in sexually mature female cobia addressed a key constraint in cobia larvae production and led to the final two studies in this dissertation. Due to issues with hormone-based spawning aids for adult cobia, a brand new excipient was tested and evaluated for adverse effects in warmwater marine species. Also, because of issues with producing fertilized cobia eggs, a live-foods-harvesting system was tested on a highly cannibalistic species. All of these experiments provide valuable insight into hatchery production.

In the evaluation of pressure- and cold-shock treatments to produce gynogens, pressure-shock was the most preferred method. This method reduced handling of embryos and led to a higher survival than did cold-shock. While both methods are economically costly, pressure-shock represents the most viable method of creating gynogenetic clones of the female that can then be sex reversed to produce a broodstock made of genetically female fish that are phenotypically male. The potential for failure within this project lies within the spawning capabilities of the female. If a female flounder does not produce viable eggs during strip spawning, treatments will have a low success rates. If the female produces viable eggs, then treatments have higher success rates.

The live-foods-harvest system did not result in larger or more numerous larval fish. However, these results were due to mechanical difficulties and fouling of the systems. As this was an original system, issues such as mechanical problems were to be expected and further testing with the live-foods-harvesting system may prove its efficiency at replacing hand-cultured live-foods for larval marine fish. Even with the negative impacts of biofouling and mechanical difficulties faced with the live-foods-harvest system, it still provides an economically feasible replacement for hand-culturing live foods and has the potential to impact hatchery production in an economically and environmentally positive manner.

Further research is needed in areas of all four of the studies undertaken to refine the methodologies so they may be used in large-scale-hatchery production. The induction of ovulation in sexually mature female cobia should be replicated using the novel hormone excipient to evaluate the effectiveness of the excipient, as well as any adverse effects it might have on such a large marine fish. The trial also should be replicated with several treatments, comparing hormone injection induced spawning with the combination or hormone-induced spawning and environmental conditioning.

In the case of the novel excipient, further trials are required to test the viability of this excipient in other species of fish. While results indicated positive initial testing of the excipient, further testing in a variety of systems on a variety of species is required. If this treatment were to be replicated, the novel excipient would be injected in several species of fish kept in replicate systems to identify adverse effects, as well as testing the

excipient on the same species in a variety of systems, such as flow through, raceway, cage, and recirculating systems.

The production of southern flounder gynogenetic clones must be expanded to include sex reversal so that all female broodstock production may be possible. This experiment also should be replicated during different stages of the lunar cycle and with females that have been evaluated for positive breeding attributes such as fecundity and repeated healthy spawns.

The live-foods-harvesting system has the potential to contribute to furthering the development of methods that potentially can be used to rear a number of new aquaculture species. However, this trial had the most equipment malfunctions. Many more replications of the live-foods-harvesting system trial must be conducted to establish the value of the system.

LITERATURE CITED

Almeida, F. L. 2013. Endocrinologia aplicada na reprodução de peixes. Revista Brasileira De Reprodução Animal 37:174–180.

Anderson, J., P. Cason, and R. Chavez. 2016. Monitoring changes in effective population size during pond culture of red drum. North American Journal of Aquaculture 79:8–17.

Animal and Plant Health Inspection Service (APHIS). 1995. Overview of Aquaculture in the United States. Centers for Epidemiology & Animal Health, United States Department of Agriculture, Fort Collins, Colorado, USA.

Arndt, R. E., E. J. Wagner, and M. E. Routledge. 2001. Reducing or withholding hydrogen peroxide treatment during a critical stage of rainbow trout development: effects on eyed eggs, hatch, deformities, and fungal control. North American Journal of Aquaculture 63:161–166.

Arnold, C. R. 1988. Controlled year- round spawning of red drum *Sciaenops ocellatus* in captivity. Contributions in Marine Science 30:65–70.

Arnold, C. R., J. B. Kaiser, and G. J. Holt. 2002. Spawning of cobia *Rachycentron canadum* in captivity. Journal of the World Aquaculture Society 33:205–208.

Arnold, C. R., J. Lasswell, W. Bailey, T. Williams, and W. Fable. 1978. Methods and techniques for spawning and rearing spotted seatrout in the laboratory. Proceedings of the Annual Conference of the Southeastern Association of Fish and Wildlife Agency 30:167–178.

Baras, E., and M. Jobling. 2002. Dynamics of intracohort cannibalism in cultured fish. Aquaculture Reserch 33:461–479.

Barnes, M. E., D. E. Ewing, R. J. Cordes, and G. L. Young. 1998. Observations on hydrogen peroxide control of *Saprolegnia* spp. during rainbow trout eggs incubation. Progressive Fish-Culturist 60:67–70.

Baroiller, J. F., Y. Guiguen, and A. Fostier. 1999. Endocrine and environmental aspects of sex differentiation in fish. Cellular and Molecular Life Sciences 55:910–931.

Benetti, D. D. 1997. Spawning and larval husbandry of flounder (*Paralichthyes woolmani*) and yellowtail (*Seriola mazatlana*), new candidate species for aquaculture. Aquaculture 155:307–318.

Benetti, D. D. 2003. Marine fish aquaculture breakthroughs in the U.S., Caribbean. Global Aquaculture Alliance Advocate 6:80–81.

Benetti, D. D., J. F. Alarcon, O. Stevens, F. Rotman, G. Banner-Steven, S. Zimmermann, M. W. Feeley, W. Matzie, M. R. Orhun, B. O'Hanlon and L. Eldridge. 2001. Marine fish culture in Latin American and Caribbean countries. Global Aquaculture Alliance Advocate 4:71–74.

Benetti, D. D., M. R. Orhun, B. O'Hanlon, I. Zink, F. G. Cavalin, B. Sardenberg, K. Palmer, B. Denlinger, and D. Bacoat. 2007. Aquaculture of cobia (*Rachycentron canadum*) in the Americas and the Caribbean. Pages 57–77 *in* I.C. Liao, and E. M Leano, editors. Cobia aquaculture: research, development, and commercial production. Asian Fisheries Society, Manilla, Philippines,

World Aquaculture Society, Louisiana, USA, The Fisheries Society of Taiwan, Keelung, Taiwan, and National Taiwan Ocean University, Keelung, Taiwan.

Benetti, D .D., M. R. Orhun, B. Sardenberg, B. O'Hanlon, A. Welch, R. Hoenig, I. Zink, J. A. Rivera, B. Denlinger, D. Bacoat, K. Palmer, and F. Cavalin. 2008. Advances in hatchery and grow-out technology of cobia *Rachycentron canadum* (Linnaeus). Aquaculture Research 39(7):701–711.

Benfey, T. J., and A. M. Sutterlin. 1984. Triploidy induced by heat shock and hydrostatic pressure in landlocked Atlantic salmon (*Salmo salar* L.). Aquaculture 36:359–367.

Biesiot, P. M., R. M. Caylor, and J. S. Franks. 1994. Biochemical and histological changes during ovarian development of cobia *Rachycentron canadum*, from the northern Gulf of Mexico. Fishery Bulletin 92:686–696.

Broach, J. S., C. L. Ohs, A. Palau, B. Danson, and D. Elefante. 2015. Induced spawning and larval culture of golden trevally. North American Journal of Aquaculture 77:532–538.

Brown-Peterson, N. J., R. M. Overstreet, J. M. Lotz, J. S. Franks, and K. M. Burns. 2001. Reproductive biology of cobia, *Rachycentron canadum*, from coastal waters of the southern United States. Fisheries Bulletin 99:15–28.

Bruno, E., J.K. Højgaard, B.W. Hansen, P. Munk, and J.G. Støttrup. 2018. Influence of swimming behavior of copepod nauplii on feeding of larval turbot (*Scophthalmus maximus*). Aquaculture International 26:225–236.

Cancino, J. M., M. Munoz, and M. C. Orellana. 1987. Effects of epifauna on algal growth and quality of the agar produced by *Gracilaria verrucosa* (Hudson) Papenfuss. Hydrobiologia 151–152:233–237.

Cason, P. D., and J. D. Anderson. 2015. A multivariate assessment of factors influencing survival of red drum (*Sciaenops ocellatus*) in earthen outdoor rearing ponds. North American Journal of Aquaculture 77:141–148.

Chesney, E. J. 1989. Estimating the food requirements of striped bass larvae *Morww saxatilis*: effects of light, turbidity and turbulence. Marine Ecology Progress Series 53:19l–200.

Chourrout, D. 1984. Pressure induced retention of 2nd polar body and suppression of 1st cleavage in rainbow trout production of all triploids all tetraploids and heterozygous and homozygous diploid gynogenetics. Aquaculture 36:111–126.

Colura, R. L., A. Henderson-Arzapalo, and A. F. Maciorowski. 1991. Culture of red drum. Pages 149–166 *in* J. P. McVey, editor. Handbook of Mariculture, Finfish Aquaculture. Volume 11. CRC Press, Boca Raton, Florida, USA.

Colura, R. L., B. R. Hysmith, and R. E. Stevens. 1976. Fingerling production of striped bass (*Marone saxatilis*), spotted sea trout (*Cynoscion nebulosus*) and red drum (*Sciaenops ocellatus*), in saltwater ponds. Proceedings of the Annual Meeting, World Mariculture Society 7(1–4):79–92.

Copeland, B. J., J. Miller, and E. B. Waters. 1998. The potential for flounder and red drum stock enhancement in North Carolina. Summary of a Workshop at North Carolina State University, Raleigh, USA.

Crim, L.W. 1985. Methods for acute and chronic hormone administration in fish. Pages 1–13 *in* C. S. Lee and L. C. Liao, editors. Reproduction and culture of milkfish. Oceanic Institute and Tungkang Marine Laboratory, Taiwan.

Cutts, C. J. 2001. Culture of harpacticoid copepods: potential as live feed for rearing marine fish. Advances in Marine Biology 44:295–316.

Czeczuga, B., R. Bartel, B. Kiziewicz, A. Godlewska, andE. Muszynska. 2005. Zoosporic fungi growing on the eggs of sea trout (*Salmo trutta* m. *trutta* L.) in river water of varied trophicity. Polish Journal of Environmental Studies 14:295–303.

Daniels, H. V., D. L. Berlinsky, R. G. Hodson, and C. V. Sullivan. 1996. Effects of stocking density, salinity, and light intensity on growth and survival of southern flounder *Paralichthys lethostigma* larvae. Journal of the World Aquaculture Society 27:153–159.

Daniels, H. V., and R. G. Hodson. 1999. Weaning success of southern flounder juveniles: effect of changeover period and diet type on growth and survival. North American Journal of Aquaculture 61:47–50.

Daniels, H. V., and W. O. Watanabe. 2003. A practical hatchery manual: production of southern flounder fingerlings. Publication No. UNC-SG-02-08, North Carolina Sea Grant, North Carolina State University, Raleigh, USA.

Das, P., S. C. Mandal, S. K. Bhagabati, M. S. Akhtar, and S. K. Singh. 2012. Important live food organisms and their role in aquaculture. Pages 69–86 *in* J. K.

Sundaray, M. Sukham, R. K. Mohanty, S. K. Otta, editors. Frontiers in aquaculture. Narendra Publishing House, New Delhi, India.

Davis, J. G. 1990. Red drum brood stock and hatchery production. Southern Regional Aquaculture Center publication 323, U.S. Department of Agriculture, Stoneville, Mississippi, USA.

DiMaggio, M. A., J. S. Broach, and C. L. Ohs. 2014. Evaluation of Ovaprim and human chorionic gonadotropin doses on spawning induction and egg and larval quality of pigfish, *Orthopristis chrysoptera*. World Aquaculture Society 45:243–257.

Dodd, Q. 2001. US cobia culture meets early success. Hatchery International 2001:14-17.

Donaldson, E. M. 1996. Manipulation of reproduction in farmed fish. Animal Reproductive Science 42:381–392.

Felip, A., S. Zanuy, M. Carrillo, and F. Piferrer. 2001. Induction of triploidy and gynogenesis in teleost fish with emphasis on marine species. Genetica 111:175–195.

Fetherman, E. R., J. M. Lepak, B. L. Brown, and D. J. Harris. 2015. Optimizing time of initiation for triploid walleye production using pressure shock treatment. North American Journal of Aquaculture 77:471–477.

Fitzhugh, G. R., L. B. Crowder, and J. P. Monaghan, Jr. 1996. Mechanisms contributing to variable growth in juvenile southern flounder (*Paralichthys lethostigma*). Canadian Journal of Fisheries Aquatic Science 53:1964–1973.

Fletcher, R. L. 1995. Epiphytism and fouling in *Gracilaria* cultivation: an overview. Journal of Applied Phycology 7:325–333.

Food and Agriculture Organization of the United Nations (FAO). 2018. The state of world fisheries and aquaculture. 2018. Meeting the sustainable development goals. Food and Agriculture Organization of the United Nations, Rome, Italy.

Franks, J. S., J. R. Ogle, J. M. Lotz, L. C. Nicholson, D. N. Barnes, and K. M. Larsen. 2001. Spontaneous spawning of cobia, *Rachycentron canadum*, induced by human chorionic gonadotropin (HCG), with comments on fertilization, hatching and larval development. Proceedings of the Gulf and Caribbean Fisheries Institute 52:598–609.

Franks, J. S., J. R. Warren, and M. V. Buchanan. 1999. Age and growth of cobia, *Rachycentron canadum*, from the northeastern Gulf of Mexico. Fishery Bulletin 97:451–479.

Fregeneda-Grandes, J. M., F. Rodriguez-Cadenas, and J. M. Aller-Gancedo. 2007. Fungi isolated from cultured eggs, alevins and broodfish of brown trout in a hatchery affected by saprolegniosis. Journal of Fishery Biology 71:510–518.

Friedlander, M, N. Galai, and H. Farbstein. 1990. A model of seaweed growth in an outdoor culture in Israel. Hydrobiologia 204–205:367–373.

Gadeken, D. L. and D. P. Casper. 2017. Evaluation of high forage total mixed ration on the locational performance of late lactation dairy cows. Translation Animal Science 1:108–115.

Garber, A. F., S. E. Fordham, J. E. Symonds, E. A. Trippel, and D. L. Berlinsky. 2009. Hormonal induction of ovulation and spermiation in Atlantic cod (*Gadus morhua*). Aquaculture 296:179–183.

Garlock, T. M., E. V. Camp, and K. Lorenzen. 2017. Using fisheries modeling to assess candidate species for marine fisheries enhancement. Fisheries Research 186:460–467.

Geiger, J. G. 1983. A review of pond zooplankton production and fertilization for the culture of larval and fingerling striped bass. Aquaculture 35:353–369.

Geiger, J. G., C. J Turner, K Fitzmayer, and W. C Nichols. 1985. Feeding habits of larval and fingerling striped bass and Zooplankton dynamics in fertilized rearing ponds. Progressive Fish-Culturist 47:213–223.

Hansen, G. H, and J. A. Olafsen. 1999. Bacterial interactions in the early life stages of marine coldwater fish. Microbial Ecology 38:1–26.

Hassler, W. W., and R. P. Rainville. 1975. Techniques for hatching and rearing cobia, *Rachycentron canadum*, through larval and juvenile stages. University of North Carolina, Publication No. UNC-SG-75-30, North Carolina Sea Grant, North Carolina State University, Raleigh, USA.

Hecht, T., and A. G. Pienaar. 1993. A review of cannibalism and its implications in fish larviculture. Journal of the World Aquaculture Society 24:246–261.

Hill, J. E., K. H. Kilgore, D. B. Pouder, J. F. F. Powell, and C. A. Watson. 2009. Survey of Ovaprim use as a spawning aid in ornamental fishes in the United

States as administered through the University of Florida. Tropical Aquaculture Lab 71:206–209.

Hoga, C. A., F. L. Almeida, and F. G. R. Reves. 2018. A review on the use of hormones in fish farming: Analytical methods to determine their residues. CyTA–Journal of Food 16:679–691.

Holt, G. J., C. K. Faulk, and M. H. Schwarz. 2007. A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish. Aquaculture 268:181–187.

Houde, E. D. 1975. Effects of stocking density and food density on survival, growth and yield of laboratory-reared larvae of sea bream *Archosargus rhomboidalis* (L.) (Sparidae). Journal of Fish Biology 7:115–127.

Houde, E .D. 1978. Critical food concentrations for larvae of three species of subtropical marine fishes. Bulletin of Marine Science 28:395–411.

Houssay, B. A. 1931. Action sexuelle de l'hypophyse sur les poissons et les reptiles. Comptes Rendus des Seances de la Societe de Biology, Paris 106:377–378.

Ibarra-Castro, L., L. Alvarez-Lajonchère, C. Rosas, I. G. Palomino-Albarrán, G. J. Holt, and A. Sanchez-Zamora. 2011. GnRHa-induced spawning with natural fertilization and pilot-scale juvenile mass production of common Snook, *Centropomus undecimali*s (Bloch, 1792). Aquaculture 319, 479–483.

Ihssen, P. E., L. R. Mckay, I. McMillan, and R. B. Phillips. 1990. Ploidymanipulation and gynogenesis in fishes: cytogenetic and fisheries applications. Transactions of the American Fisheries Society 119:698–717.

Ingram, B., S. Sungan, G. Gooley, S. Y. Sim, D. Tinggi, and S. S. De Silva. 2005. Induced spawning, larval development and rearing of two indigenous Malaysian mahseer, *Tor tambroides* and *T. douronensis*. Aquaculture Research 36:983–995.

Jackson, J. M., and P. H. Lenz. 2016. Predator-prey interactions in the plankton: larval fish feeding on evasive copepods. Scientific Reports 6:33585.

Kaiser, J. B., and G. J. Holt. 2005. Species profile: cobia. Publication 7202, Southern Regional Aquaculture Center, Stoneville, Mississippi, USA.

Kilduff, P., W. DuPaul, M. Oesterling, J., Jr. Olney, and J. Tellock. 2002. Induced tank spawning of cobia, *Rachycentron canadu*m, and early larval husbandry. World Aquaculture 33:35–39.

King, N. J., G. C. Nardi, and C. J. Jones. 2001. Sex-linked growth divergence of southern flounder from a commercial farm: are males worth the effort? Journal of Applied Aquaculture 11:77–88.

Kolkovski, S, A. Arieli, and A. Tandler. 1997. Visual and chemical cues stimulate microdiet ingestion in sea bream larvae. Aquaculture International 5:527–536.

Kolkovski, S, J. Curnow, and J. King. 2004. Intensive rearing system for fish larvae research II Artemia hatching and enriching system. Aquacultural Engineering 31:309–317.

Kuradomi, R. Y., F. Foresti, and S. R. Batlouni. 2017. The effects of sGnRHa implants on *Piaractus mesopotamicus* female breeders. An approach addressed to aquaculture. Aquaculture International 25:2259–2273.

Lam, T. J. 1982. Applications of endocrinology to fish culture. Canadian Journal of Fisheries and Aquatic Sciences 39:111–137.

Lee, C. -S. 1997. Advanced technology in sustainable marine finfish culture: fry production. Pages 48–58 *in* K. P. P. Nambiar and T. Singh, editors. Sustainable aquaculture, Proceedings of the INFOFISH-AQAUATECH 96: international conference on aquaculture, Kuala Lumpur, Malaysia.

Lee, C. -S., and A. C. Ostrowski. 2001. Current status of marine finfish larviculture in the United States. Aquaculture 200:89–109.

Lee, C. -S., C. S. Tamaru, J. E. Banno, and C. D. Kelley. 1986*a*. Influence of chronic administration of LHRH-analogue and/or 17 α-methyltestosterone on maturation in milkfish, *Chanos chanos*. Aquaculture 59:147–159.

Lee, C.-S., C.S. Tamaru and C.D. Kelley. 1986*b*. Technique for making chronic-release LHRH-a and l 7a-Methyltestosterone pellets for intramuscular implantation in fishes. Aquaculture 59:161–168.

Lee, C. -S., C. S. Tamaru, and C.D. Kelley. 1988. The cost and effectiveness of CPH, HCG and LHRH-a on the induced spawning of grey mullet, *Mugil cephalus*. Aquaculture 73:341–347.

Lia, R. P., S. Rehbein, A. Giannelli, B. Fankhauser, and D. Otranto. 2019. LONGRANGE® (eprinomectin 5% w/v extended-release injection) efficacy against *Hypoderma lineatum* in an endemic area in southern Italy. Parasites & Vectors 12:231.

Liao, I., T. Juang, W. Tsia, C. Hsueh, S. Chang, and E. Leano. 2004. Cobia culture in Taiwan: current status and problems. Aquaculture 237:155–165.

Lim, C. L., P. Dhert, and P. Soregloos. 2003. Recent developments in the application of live feeds in the freshwater ornamental fish culture. Aquaculture 227:319–331.

Lipscomb, T. N., A. L. Wood, M. A. DiMaggio, Q. M. Tuckett, L. L. Lawson, and C. A. Watson. 2018. Evaluation of spawning aids and administration routes on ovulation success in an ornamental cyprinid. Aquaculture Research 49:3926–3929.

Luckenbach, J. A., J. Godwin, H. V. Daniels, J. M. Beasley, C. V. Sullivan, and R. J. Borski. 2004. Induction of diploid gynogenesis in southern flounder (*Paralichthys lethostigma*) with homologous and heterologous sperm. Aquaculture 237:499–516.

Luckenbach, J. A., J. Gowdwin, H. V. Daniels, and R. J. Borski. 2003. Gonadal differentiation and effects of temperature on sex determination in southern flounder (*Paralichthys lethostigma*). Aquaculture 216:315–327.

Lyon, D. D., and M. R. Fisher. 1998. Temperature and pH-related mortality of red drum larvae in fertilized culture ponds. Progressive Fish-Culturist 60:227–230.

Manley, C. B., C. F. Rakocinski, P. G. Lee, and R. B. Blaylock. 2014. Stocking density effects on aggressive and cannibalistic behaviors in larval hatchery- reared spotted seatrout, *Cynoscion nebulosus*. Aquaculture 420–421:89–94.

Migaud, H., G. Bell, E. Cabrita, B. Mcandrew, A. Davie, and J. Bobe. 2013. Gamete quality and broodstock management in temperate fish. Review of Aquaculture 5:194–223.

Moffatt, N. M. 1981. Survival and growth of northern anchovy larvae on low zooplankton densities as affected by the presence of a *Chlorellu* bloom. Rapports et Proces Verbaux des Reunions Conseil International pour l'Exploration de la Mer 178:475–480.

Monaghan, J. P., and J. L. Armstrong. 2000. Reproductive ecology of selected marine recreational fishes in North Carolina. Completion Report Grant F-60, North Carolina Department of Environment and Natural Resources, Division of Marine Fisheries, Morehead City, USA.

Morgan, A. J., R. Murashige, C. A. Woolridge, J. A. Luckenbach, W. O. Watanabe, R. J. Borski, J. Godwin, and H. V. Daniels. 2006. Effective UVdose and pressure shock for induction of meiotic gynogenesis in southern flounder (*Paralichthys lethostigma*) using black sea bass (*Centropristis striata*) sperm. Aquaculture 259:290–299.

Murugananthkumar, R., Y. Prathibha, B. Senthilkumaran, A. Rajakumar, and H. Kagawa. 2017. In vivo induction of human chorionic gonadotropin by osmotic pump advances sexual maturation during pre-spawning phase in adult catfish. General and Comparative Endocrinology 251:78–84.

Mussi, M, W. N. McFarland and P. Domenici. 2005. Visual cues eliciting the feeding reaction of a planktivorous fish swimming in a current. Journal of Experimental Biology 208:831–842.

Mylonas, C. C., A. Fostier, and S. Zanuy. 2010. Broodstock management and hormonal manipulations of fish reproduction. General and Comparative Endocrinology 165:516–534.

Nakamura, M., T. Kobayashi, X. Chang, and Y. Nagahama. 1998. Gonadal sex differentiation in teleost fish. Journal of Experimental Zoology 281:362–372.

Naas, K. E., T. Næss, and T. Harboe. 1992. Enhanced first feeding of halibut larvae (*Hippoglossus hippoglossus* L.) in green water. Aquaculture 105:143–156.

Nelson, G. R., M. R. DeVoe, and G. L. Jensen. 1999. Status, experience, and impacts of state aquaculture plans and coastal zone management plans on aquaculture in the United States. Journal of Applied Aquaculture 9:1–21.

New, M. B. 1999. Global aquaculture: current trends and challenges for the 21st century. World Aquaculture 30:8–14.

Nguyen, H. Q., T. M. Tran, H. Reinertsen, and E. Kjørsvik. 2010. Effects of dietary essential fatty acid levels on broodstock spawning performance and egg fatty acid composition of cobia, *Rachycentron canadum*. Journal of the World Aquaculture Society 41:687–699.

NOAA Fisheries. 2013. Fisheries of the United States, 2013. NOAA current fishery statistics No.2013, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, Washington D.C., USA.

NOAA Fisheries. 2015. 2014 Aquaculture production. NOAA Fisheries, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, Washington, D.C., USA.

NOAA Fisheries. 2018. Fisheries of the United States, 2017 report. NOAA Fisheries, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, Washington, D.C., USA.

Olafsen, J. A. 2001. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200:223–247.

Pandian, T. J., and R. Koteeswaran. 1999. Lability of sex differentiation in fish. Current Science 76:580–583.

Person Le Ruyet, J., J.C. Alexandre, L. Th baud, and C. Mugnier. 1993. Marine fish larvae feeding: formulated diets or live prey? Journal of the World Aquaculture Society 24:211–224.

Peruzzi, S., and B. Chatain. 2000. Pressure and cold shock induction of meiotic gynogenesis and triploidy in the European sea bass, *Dicenlrarchus labr*ax L: relative efficiency of methods and parental variability. Aquaculture 189:23–37.

Peruzzi, S., A. G. Scott, J. C. J. Domaniewski, and G.F. Warner. 1993. Initiation of gynogenesis in *Oreochromis niloticus* following fertilization. Journal of Fisheries Biology 43:585–591.

Purdom, C. E. 1969. Radiation-induced gynogenesis and androgenesis in fish. Heredity 24:431–44.

Roberts, A. J., and M. R. Conover. 2013. Eared grebe diet on Great Salt Lake, Utah, and competition with the commercial harvest of brine shrimp cysts. Journal of Wildlife Management 77:1380–1385.

Roberts, A. J., and M. R. Conover. 2014. Diet and body mass of ducks in the presence of commercial harvest of brine shrimp cysts in the Great Salt Lake, Utah. Journal of Wildlife Management 78:1197–1205.

Rottmann, R. W., J. V. Shireman, and F. A. Chapman. 1991. Hormonal control of reproduction in fish for induced spawning. Southern Regional Aquaculture Center Publication No. 424, Stoneville, Mississippi, USA.

Rueness, J, and S. Fredriksen. 1989. Culture studies of agarophytes with special reference to Gelidium species at their northern limit of distribution. Pages 55–58 *in* J. M. Kain, J. W. Andrews, and B. J. McGregor, editors. Out-door seaweed cultivation. Proceedings of the Second Workshop of COST 48, Subgroup 1. European Economic Community, Brussels, Belgium.

Sandifer, P. A., J. S. Hopkins, A. D. Stokes, and R. D. Smiley. 1993. Experimental pond grow-out of red drum, *Sciaenops ocellatus*, in South Carolina. Aquaculture 118:217–228.

Sapkota, P., C. Engle, D. Heikes, and N. Stone. 2012. Cost analysis of a mobile fish nursery system for growing hybrid striped bass (*Morone chrysops* x *M. saxatilis*) fry. Aquacultural Engineering 49:18–22.

Schally, A. V. 1978. Aspects of hypothalamic regulation of the pituitary gland. Science 202:18–28.

Schwarz, M. H. 2004. Fingerlings production still bottleneck for cobia culture. Global Aquaculture Advocate 7:40–41.

Sherwood, N. M., L.W. Crim, J. Carolsfeld and S. M. Walters. 1988. Sustained hormone release. I. Characteristics of in vitro release of gonadotropin-releasing hormone analogue (GnRH-A) from pellets. Aquaculture 74:75–86.

Sink, T. D., R. J. Strange, and R. T. Lochmann. 2010. Hatchery methods and natural, hormone-implant-induced, and synchronized spawning of captive Atlantic croaker (*Micropogonias undulatus*) Linnaeus 1766. Aquaculture 307:35–43.

Sink, T., R. Vega, and J. Butler. 2018. Red drum: reproductive biology, broodstock management, and spawning. Southern Regional Aquaculture Center, Publication No. 0320, Stoneville, Mississippi, USA.

Smith, A. H., K. Nichols, and J. McLachlan. 1984. Cultivation of seamoss (Gracilaria) in St Lucia, West Indies. Hydrobiologia 116/117:249–251.

Smith, T. I. J., D. C. McVey, W. E. Jenkins, M. R. Denson, L. D. Heyward, C. V. Sullivan, and D. L. Berlinsky. 1999. Broodstock management and spawning of southern flounder, *Paralichthys lethostigma*. Aquaculture 176:87–99.

Stieglitz, J. D., D. D. Benetti, R. H. Hoenig, B. Sardenberg, A. W. Welch, and S. Miralao. 2012. Environmentally conditioned, year-round volitional spawning of cobia (*Rachycentron canadum*) in broodstock maturation systems. Aquaculture Research 43:1557–1566.

Su, M.S., Y. H. Chien, and I. C. Liao. 2000. Potential of marine age aquaculture in Taiwan: cobia culture. Pages 97–106 *in* I. C. Liao and C. K. Lin, editors. Cage aquaculture in Asia. Asian Fisheries Society, Manila, Philippines and World Aquaculture Society-Southeast Asian Chapter, Bangkok, Thailand.

System for Electronic Document Analysis and Retrieval (SCDNR). 2015. Stocking red drum larvae in the Ashley River. South Carolina Department of Natural Resources, Columbia, USA.

System for Electronic Document Analysis and Retrieval (SEDAR). 2013. SEDAR 28– Gulf of Mexico cobia stock assessment report. North Charleston, South Carolina, USA.

Tamaru, C. S., C. D. Kelley, C. -S. Lee, K. Aida, and I. Hanyu. 1989. Effects of chronic LHRH-a+17α-methyltestosterone or LHRH-a+Testosterone therapy on vitellogenesis in the striped mullet (*Mugil cephalus*). General and Comparative Endocrinology 76:114–127.

Torati, L. S., H. Migaud, M. K. Doherty, J. Siwy, W. Mullen, P. E. C. Mesquita, and A. Albalat. 2017. Comparative proteome and peptidome analysis of the cephalic fluid secreted by *Arapaima gigas* (Teleostei: Osteoglossidae) during and outside parental care. PLoSONE12 (10):e0186692.

Tucker, J. W., Jr. 1988. Growth of juvenile spotted seatrout on dry feeds. Progressive Fish-Culturist 50:39–41.

Tucker, J. W., Jr, P. N. Woodward, and D. G. Sennett. 1996. Voluntary spawning of captive Nassau groupers *Epinephelus striatus* in a concrete raceway. Journal of the World Aquaculture Society 27:373–383.

Tvedt, H. B., T. J. Benfey, D. J. Martin-Robichaud, and J. Power. 2001. The relationship between sperm density, spermatocrit, sperm motility and fertilization

success in Atlantic halibut, *Hippoglossus hippoglossus*. Aquaculture 194:191–200.

United States Department of Agriculture (USDA). 2013. 2012 Census of Aquaculture. Volume 3. Special Studies Part 2, AC-12-SS-2, United States Department of Agriculture, Washington, D.C., USA.

Valentinčič, T., D. F. Lamp, and J. Caprio. 1999. Expression of a reflex biting/snapping response to amino acids prior to first exogenous feeding in salmonid alevins. Physiological Behavior 67:567–572.

Vazirzadeh, A, B. M. Amiri, S. Yelghi, A. Hajimoradloo, M. A. Nematollahi, and C. C. Mylonas. 2011. Comparison of the effects of different methods of mammalian and salmon GnRHa administration on spawning performance in wild-caught female carp (*Cyprinus carpio carpio*) from the Caspian Sea. Aquaculture, 320:123–128.

Vega-Ramirez, M. T., M. C. Moreno-Lafont, R. Valenzuela, R. A. Cervantes-Olivares, J. M. Aller-Gancedo J. M. Fregeneda-Grandes, J. L. Damas-Aguilar, V. Garcia-Flores, and R. Lopex-Santiago. 2013. New records of Saprolegniaceae isolated from rainbow trout, from their eggs, and water in a fish farm from the State of Mexico. Revista Mexicana de Biodiversidad 84:637–649.

Venturieri, R., and G. Bernardino. 1999. Hormônios na reprodução artificial de peixes (Hormones in artificial fish breeding). Panorama Da Aqüicultura 9(55):39–48.

von Ihering, R. 1937. A method for inducing fish to spawn. Progressive Fish Culturist 34:15–16.

Watanabe, W. O., P. M. Carroll, and H. V. Daniels. 2001. Sustained, natural spawning of southern flounder *Paralichthys lethostigma* under an extended photothermal regime. Journal of the World Aquaculture Society 32:153–166.

Watanabe, W. O., C. A. Woolridge, and H. V. Daniels. 2006. Progress toward year-round spawning of southern flounder broodstock by manipulation of photoperiod and temperature. Journal of the World Aquaculture Society 37:256–272.

Weirich, C. R., T. I. J. Smith, M. R. Denson, A. D. Stokes, and W. E. Jenkins. 2004. Pond culture of larval and juvenile cobia, *Rachycentron canadum*, in the southeastern United States: initial observations. Journal of Applied Aquaculture 16:27–44.

Weirich, C. R., A. D. Stokes, T .I. J. Smith, W. E. Jenkins, and M. R. Denson. 2006. Outdoor tank and pond spawning of cobia, *Rachycentron canadum*, in coastal South Carolina. Journal of Applied Aquaculture 18:1–16.

White, K., B. N. O'Neill, and Z. Tzankova. 2004. At a crossroads: will aquaculture fulfill the promise of the blue revolution? Report, Sea Web Aquaculture Clearinghouse, Washington, D.C., USA.

Wisner, B. B., K. L. Mertz, and T. R. Capo. 1996. Spawning frequency and egg production of captive spotted seatrout. Progressive Fish Culturist 58:140–142.

Yamamoto, E. 1995. Studies on sex-manipulation and production of cloned populations in hirame flounder, *Paralichthys olivaceus*. Bulletin of the Tottori Prefectural Fisheries Experimental Station 34:1–145 (in Japanese, with English summary).

Yamamoto, E. 1999. Studies on sex-manipulation and production of cloned populations in hirame, *Paralichthys olivaceus*. Aquaculture 173:235–246.

Zarela, A. 2010. Artemia meal: a newer animal protein source for poultry. Indian Journal of Animal Research, 44:235–240.

Zohar, Y., and C. C. Mylonas. 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 197:99–136.